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# A Rapid *in Vitro* Assay for Quantitating the Invasive Potential of Tumor Cells

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## ABSTRACT

We have reconstituted a matrix of basement membrane onto a filter in a Boyden chamber and assessed the ability of various malignant and nonmalignant cells to penetrate through the coated filter. Cells from all the malignant cell lines tested were able to cross the matrix in 5–6 h, whereas human fibroblasts as well as mouse 3T3 and 10T½ cell lines, which are not tumorigenic, were not invasive. In addition, normal primary prostate epithelial cells and benign prostatic hyperplasia cells were not invasive when tested in this assay, whereas malignant prostate carcinoma cells were highly invasive. Parallel experiments with these prostatic cells using the intrasplenic assay for metastasis detection in the nude mouse confirmed the benign behavior of the former cells and the metastatic phenotype of the latter ones. These results suggest that this *in vitro* test allows the rapid and quantitative assessment of invasiveness and a means to screen for drugs which alter the invasive phenotype of tumor cells.

## INTRODUCTION

Basement membranes are thin continuous sheets which separate epithelial tissues from adjacent stroma (1). The predominant components of basement membranes are laminin, a large multifunctional glycoprotein, collagen IV, and a heparan sulfate proteoglycan (2, 3). Additional common components have been identified, as well as other components with a more limited distribution (4, 5). Basement membranes form barriers that block the passage of cells and macromolecules but become permeable during tissue development and repair, at inflammatory sites, and are resorbed in areas where basement membranes contact invasive neoplasms (6). Tumor invasion of basement membranes is a crucial step in the complex multistage process which leads to the formation of a metastasis (6). Tumor cells cross basement membranes as they initially invade the lymphatic or vascular beds during dissemination, and when they penetrate into their target tissue (7). It seems likely that the penetration of the tumor cell into basement membranes involves distinct events which include: (*a*) attachment of the tumor cells to the basement membrane via cell surface receptors (8–12); (*b*) secretion of enzymes by the tumor cells that cause the degradation of the adjacent basement membrane (9, 13–15); and (*c*) migration of the cells into the target tissue in response to specific chemotactic stimuli (16–19).

A variety of *in vitro* systems have been developed to assess the invasiveness of tumor cells (for a review, see Ref. 20). Several of these assays utilize tissues which contain basement membranes, such as bladder wall (21), amnion (22), lens capsule (23), and chick chorioallantoic membranes (24). In addition, a reconstituted disc composed of lyophilized collagen IV and laminin has also been used to assay for the invasiveness of tumor cells (4) in Boyden chambers. The behavior of human HT-1080 fibrosarcoma cells on reconstituted basement membrane (*i.e.*, matrigel) (5) in Petri dishes has recently been reported (26). The cells were found to invade this matrix, leaving tunnels and passages and ultimately degrading the gel.

Here we have used matrigel as a coating on the top of a porous filter as a barrier in Boyden chambers to test the invasive behavior of tumor cells. Fibroblast-conditioned medium, which contains known (*i.e.*, fibronectin and collagen) as well as unknown chemotactic factors (27), was placed in the lower well beneath the filter to stimulate rapid penetration of the cells. Some 20 normal and tumorigenic cell lines were studied and the malignant cells were found to be the more invasive. Melanoma cells recovered after invading the matrix were found to be over 2-fold more invasive when retested in the assay, probably due to the selection of a more invasive subpopulation of cells. Striking differences in morphology were noted between malignant and nonmalignant cells cultured on the matrigel.

## MATERIALS AND METHODS

**Cell Lines.** Murine melanoma cell lines B16F1 and B16F10 were provided by I. J. Fidler, Houston, TX. B16F1 cells are a subline with low metastatic potential and B16F10 cells are a high metastatic variant of the same tumor (28). $B16Br_2$ is a line of melanoma cells derived from a brain metastasis, which is highly invasive *in vivo* and *in vitro* (25). Two lines derived from the K-1735 UV-induced melanoma, C110 (nonmetastatic) (29) and M2 (highly metastatic) (30), were also provided by I. J. Fidler.

Nontumorigenic cells, MRC-5 (31) human embryonic fibroblasts (CCL 171), and C3H/10T½ (32) mouse fibroblasts (CRL 226), were obtained from the American Type Culture Collection. Several cell lines known to form malignant tumors *in vivo* were also obtained from the American Type Culture Collection: ME-180 (HTB 33), an invasive squamous cell epidermoid carcinoma of the ovary which had metastasized to the omentum (33); Malme 3M (HTB 64), a lung metastasis from a human melanoma (34); A204 (HTB 82), a human rhabdomyosarcoma (35); SW 620 (CCL 228), a grade III–IV colon adenocarcinoma (36); and PA-1 (CCL 1572), an ovarian teratocarcinoma (37) adapted to ascitic culture. The line HT-1080 (CCL 121) was derived from a metastatic lesion of a human fibrosarcoma (38). All cells were maintained in DMEM[2] supplemented with 10% fetal calf serum, glutamine, vitamins, nonessential amino acids, and antibiotics.

T24/3T3 cells were derived upon transfection of NIH 3T3 cells with DNA from the human bladder carcinoma T24, and contain an activated H-*ras* oncogene with a point mutation in the position corresponding to amino acid 12 (39, 40). Hs242/3T3 were obtained by transfecting NIH 3T3 cells with DNA of the human lung carcinoma Hs242, and contained an H-*ras* oncogene with a mutation in position 61 (41). NIH 3T3 cells and their transfectants were maintained in medium supplemented with 10% calf serum, glutamine, and antibiotics.

Normal prostate epithelial cells, and benign prostate hyperplasia cells were obtained at surgery and were isolated by collagenase digestion. Du 145 cells are a human prostate carcinoma cell line established by Stone *et al.* (42), which only rarely gives rise to metastasis *in vivo*[3]; Du LM is derived from the rare liver metastasis formed from Du 145 cells injected in nude mice.[3] PC 3 cells are a hormone-resistant line derived from a bone metastasis of a prostatic cancer (43). Primary prostate epithelial cells were grown in WAJC 404 medium (44) (Irvine Scientific, Santa Ana, CA), supplemented with glutamine, antibiotics, and the following factors: epidermal growth factor (10 ng/ml); bovine pituitary extract (30 μg/ml); insulin, transferrin, and selenium in linolenic acid and bovine serum albumin (10 ml/liter) (Collaborative Re-

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[2] The abbreviation used is: DMEM, Dulbecco's minimal essential medium.
[3] J. M. Kozlowski *et al.*, unpublished data.

search, Waltham, MA); cholera toxin (10 ng/ml); prolactin (2 μg/ml); and 2 mg/ml polyvinylpyrrolidone ($M_r$ 40,000) (Sigma Chemical Co., St. Louis, MO). DU 145 and Du LM cells were maintained in DMEM supplemented with 4.5 g of glucose/liter, nonessential amino acids, and 10% fetal bovine serum. PC 3 cells were grown in RPMI 1640 supplemented with glutamine and 10% fetal bovine serum.

Basement Membrane Matrigel. The basement membrane matrigel, an extract of the Englebreth-Holm-Swarm tumor, was prepared in sterile form by exposure to chloroform and then dialyzed as previously described (5). The amount of protein in the gel was estimated according to Lowry *et al.* (45). The concentration of protein in the batch used throughout these studies was 10 mg/ml.

Chemoinvasion Assay. Blind well chemotaxis chambers with 13-mm diameter filters were used for the assay (Fig. 1). Polyvinylpyrrolidone-free polycarbonate filters, 8-μm and 12-μm pore size (Nucleopore, California), were coated with varying amounts of basement membrane matrigel (12.5-200 μg/filter). The matrigel was diluted to the desired final concentration with cold distilled water, applied to the filters, dried under a hood, and reconstituted with serum-free medium. In some cases, the homogeneity of the coating was checked by protein stain. The coated filters were placed in Boyden chambers. Cells (2-3 × $10^5$), suspended in DMEM containing 0.1% bovine serum albumin, were added to the upper chamber. Under these conditions, few cells died within 24 h as measured by trypan blue exclusion in preliminary experiments.

Conditioned medium was obtained by incubating either mouse or human fibroblasts for 24 h in serum-free medium in the presence of ascorbate. Comparable results were obtained using either source of conditioned medium in preliminary experiments and for convenience 3T3-conditioned medium was used here. This medium was used as a source of chemoattractants and was placed in the lower compartment of the Boyden chambers. There is very little cell migration (less than 5%) in the absence of the chemoattractant over a 6-h period, and no passive diffusion since the pores of the filter are smaller than the cells. Assays were carried out at 37°C in 5% $CO_2$. The gradient of chemotactic protein is stable for at least 8-10 h as determined by the diffusion of radiolabeled proteins.[4] Over 90% of the cells attach to the filter after a 2-h incubation. At the end of the incubation, the cells on the upper surface of the filter were completely removed by wiping with a cotton swab, as monitored visually under high power. The filters were fixed in methanol and were stained with hematoxylin and eosin. Cells from various areas of the lower surface were counted and each assay was performed in triplicate.

Chemotaxis. For chemotaxis studies, filters were coated with collagen type IV alone (5 μg/filter) to promote cell adhesion. Fibronectin (25 μg/ml), conditioned medium, and medium alone were used as chemoattractants in the lower compartment of the chamber. Cells were added to the upper chamber and the assay was carried out as previously described (27).

~~Recovery of Invasive~~ Cells. In some cases, these assays were carried out under sterile conditions to allow recovery of the invasive cells. The chambers were autoclaved and placed inside sterile Petri dishes (46). The uncoated filters were sterilized by UV. All solutions were sterile and the assembly of the chambers was carried out in a sterile hood. Two filters were used, including an 8-μm filter coated with matrigel and a second one with very small pores (1 μm) which was placed below it to retain the cells that passed the first filter. The second filter was coated with collagen IV to promote cell adhesion and the retention of cells on its surface. After 5 h, the matrigel and upper filter were removed. The lower filter, which contained the "invasive" cells, was rinsed and placed in a 13-mm tissue culture well containing complete culture medium. The cells were allowed to grow and were subsequently passaged and reassayed for invasive behavior. Unselected control cells were assayed at the same passage number as the selected cells.

Morphology of Tumor Cells on Matrigel. The appearance of prostate cells cultured on the matrigel was assessed. Matrigel (0.5 ml/16-mm-diameter tissue culture dish well, i.e., 5 mg of matrigel/dish) was polymerized at 37°C for 30 min. A cell suspension (usually 5 × $10^4$ cells/well) in DMEM plus 10% fetal bovine serum was pipeted onto the top of the gel. Cells were photographed after 2-3 days.

*In Vivo* Assessment of Metastatic Activity. The metastatic capability of prostate-derived cells *in vivo* was assessed in nude mice by the intrasplenic assay (47). Cells (5 × $10^5$, viability over 95%) were injected into the medial tip of the spleen of mice anesthetized with methoxyfluorane (Metofane). No significant bleeding or extravasation of cells was noted. The animals were killed by cervical dislocation 8 weeks after injection of the cells. Autopsies were performed and the organs (spleen, liver, foregut and hindgut mesentery, and lungs) were fixed, embedded in paraffin, and examined histologically.

## RESULTS

To determine which conditions would allow a rapid but discriminating assay, we varied the amount of basement membrane matrigel placed on the filter and assessed the penetration of normal and malignant cells as a function of time. These studies showed that 50 μg of matrigel formed a continuous thin even barrier (Fig. 1) covering the surface of the filter but barely entering the pores. Few cells were found to penetrate through the matrigel during the first 3 h of the assay (Fig. 2A). After 5 and 6 h, many more cells from the malignant cell lines than from the nonmalignant controls were found to penetrate through the matrigel and filter. When lower levels of matrigel were used as a coating, more of the control cells penetrated and there was less of a difference between the malignant and control cells. Few cells were found to invade over a 5-h period when the amount of matrigel was increased to 100 μg/filter (Fig. 2B). However, a large number of malignant cells were able to invade through 100-μg matrigel in 24 h and through 200 μg after 48 h (data not shown). These observations indicate that the time for the invasive cells to pass through the barrier is proportional to the amount of matrix on the filter. For convenience, we selected a 5-h assay with 50 μg of matrigel on the filter for further investigation.

Various cells of known metastatic potential were examined in the invasion assay to determine if their ability to penetrate the reconstituted basement membrane matrigel correlated with their *in vivo* history. Indeed, those cell lines known to be malignant showed a higher rate of invasion than nonmetastatic tumor cells and the control cells showed little or no ability to penetrate the barrier (less than 2 cells/field) (Table 1). For example, various fibroblastic cell lines and a nonmetastatic clone (Cl10) from the K1735 mouse melanoma tumor did not invade the reconstituted matrix, whereas cells from several highly metastatic lines, including SW 620, a colon adenocarcinoma, and PA-1 teratocarcinoma and a metastatic clone (M2) from the K1735 tumor were highly invasive. In addition, the low metastatic line B16F1 of the B16 mouse melanoma demonstrated lower invasive activity than the more metastatic B16F10 line (22 cells/field *versus* 40 cells/field). B16$Br_2$ cells, obtained from a brain metastasis, were even more invasive (102 cells/field). We also studied B16$Br_2$ cells which had been collected after they had penetrated once through the reconstituted basement membrane and retested them in the invasion assay. These "selected" cells were more than twice as invasive *in vitro* as the parent line. Thus, this *in vitro* procedure not only characterizes the ability of cells to invade a basement membrane matrix but also allows the isolation of those cells which have successfully crossed the matrix barrier.

The chemotactic response of the cells was also tested to determine if the failure of the nonmetastatic tumor cells and normal cells to invade was due to their lacking this response. Indeed, all the normal cells tested here plus the nonmetastatic tumor lines showed a good chemotactic response (data not

[4] A. Albini and B. Adelmann-Grill, unpublished data.

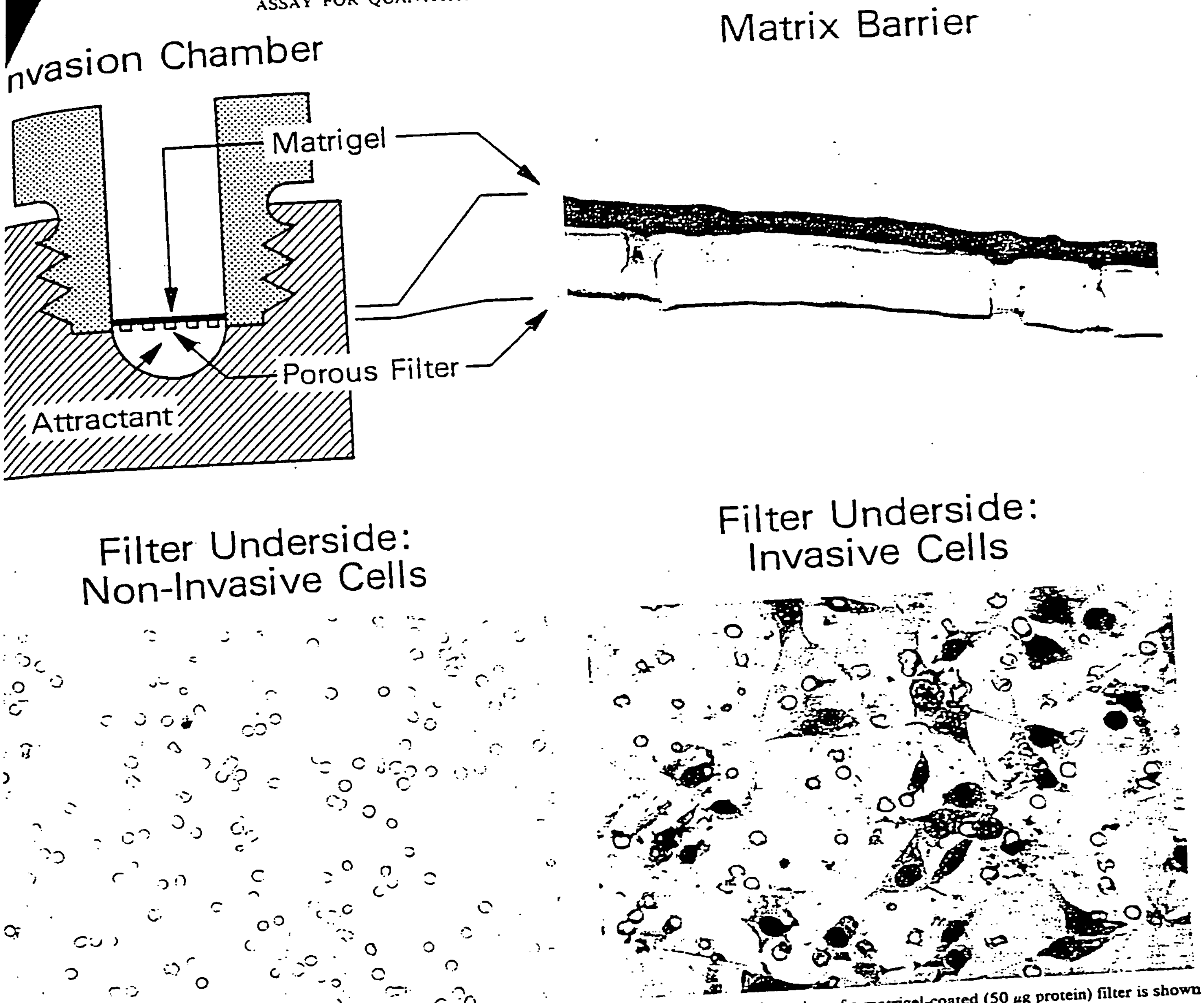


Fig. 1. Invasion assay: Boyden chamber assembly used for the invasion assay is shown in the *upper left*; section of a matrigel-coated (50 $\mu g$ protein) filter is shown in the *upper right*; lower surfaces of an invasion membrane when NIH 3T3 (*lower left*) and T24/3T3 (*lower right*) are assayed for invasion.

shown). When matched cell lines were examined, such as 3T3 and 3T3 transfected with *ras* (Fig. 3) and the invasive and noninvasive lines from the 1735 melanoma clones C110 and M2 (data not shown), we found that the chemotactic response of the malignant cell was somewhat greater than its control line (1.5 to 2 times), but that invasiveness was some 15-fold greater. These results indicate that both the noninvasive and invasive cells are able to migrate to chemoattractants and that the basement membrane represents the critical barrier in our assay.

Studies on Prostate Cells. We also tested several human prostatic epithelial cell lines for their invasive activity *in vitro* and for their metastatic capability *in vivo*. These cells were studied because they represent a graded series differing in their malignancy in the human and in their metastatic abilities in nude mice. Normal prostatic cells did not invade the basement membrane matrigel nor form metastases in the nude mouse (data not shown). Primary benign hyperplastic prostatic cells, which did not form tumors in the animal after intrasplenic assay, were not invasive (Table 2). The prostatic carcinoma line (Du 145) gave rise to local tumors but showed base-line levels of invasiveness *in vitro* and very little metastatic activity *in vivo*. In contrast, cells from the rare liver metastasis of the Du 145 line, designated Du LM, and a highly malignant prostate tumor line, PC 3, invaded the matrix and also formed metastases *in vivo*. The organs involved were liver, parietal peritoneum, mesenteric and mediastinal lymph nodes, and lung parenchyma. This suggests that the *in vitro* invasion assay may supplement the intrasplenic assay for detecting metastatic variants and for quantitating metastatic potential.

No morphological differences were noted in the appearance of the various lines of human prostate cells whether normal or malignant when cultured on plastic substrates. However, when plated onto a matrigel substrate, the cells showed striking differences. Under these conditions, the normal cells formed secretory domes and the low-grade carcinoma cells aggregated into tight colonies. In contrast, the metastatic lines extended, spread, and formed branching, invasive colonies (Fig. 4, *right*). This distinctive behavior was apparent after 24 to 48 h in

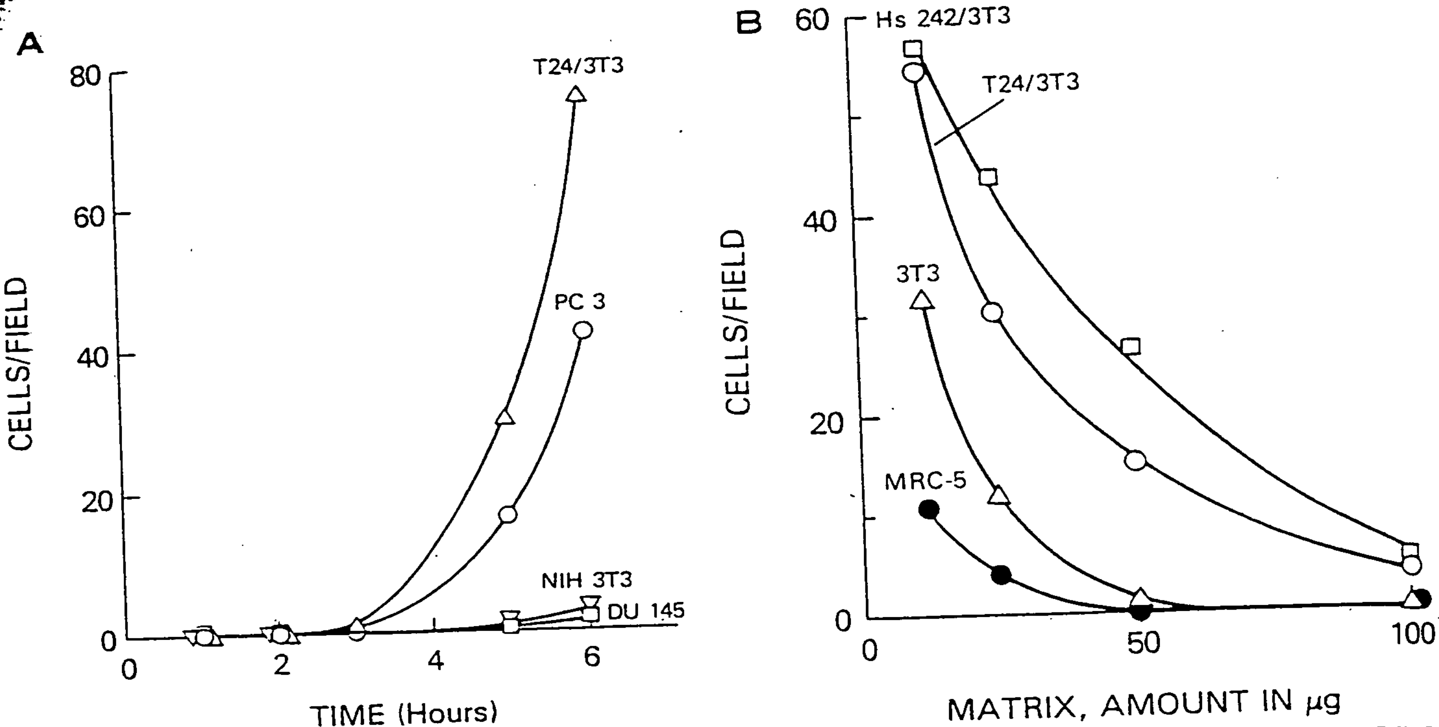


Fig. 2. *A*, time dependence of the invasion of the matrigel-coated filters for nonmalignant (NIH 3T3 and Du 145) and malignant lines (T24/3T3 and PC 3). *B*, dependence of varying amounts of matrigel coating on the invasiveness of nonmalignant (NIH-3T3 and MRC-5) and malignant (T24/3T3 and Hs 242/3T3) lines.

Table 1 *Invasiveness of various cell lines*

| Cell line | Origin | Malignancy *in vivo* | Invasiveness *in vitro* (cells/field) |
|---|---|---|---|
| Human | | | |
| MRC-5 | Embryonic fibroblast | No | 0[a] (15)[b] |
| HT1080 | Fibrosarcoma | Yes | 8 (9) |
| ME-180 | Cervical carcinoma (metastasis) | Yes | 14 (6) |
| Malme 3M | Melanoma (metastasis) | Yes | 14 (3) |
| A 204 | Rhabdomyosarcoma | Yes | 28 (3) |
| SW 620 | Colon adenocarcinoma (metastasis) | Yes | 30 (6) |
| PA-1 | Ovary teratocarcinoma (ascites) | Yes | 38 (24) |
| Mouse | | | |
| C3H/10T½ | Fibroblastoid | No | 2 (15) |
| NIH 3T3 | Fibroblastoid | No | 2 (24) |
| T24/3T3 | Ha-*ras*-transfected 3T3 | Yes | 30 (18) |
| HS242/3T3 | Ha-*ras*-transfected 3T3 | Yes | 44 (12) |
| K 1735 C110 | Melanoma | No | 4 (30) |
| K 1735 M2 | Melanoma | Yes (high) | 44 (30) |
| B16F1 | Melanoma | Yes (low) | 22 (9) |
| B16F10 | Melanoma | Yes (high) | 40 (9) |
| $B16Br_2$ | Melanoma | Yes (high) | 102 (24) |
| $Br_2$ MG | $B16Br_2$, matrigel selected | ND[c] | >200 (6) |

[a] Data are expressed as migrated cells/field (×160).
[b] Numbers in parentheses, number of assays run for each line. SD was less than 10%.
[c] ND, not determined.

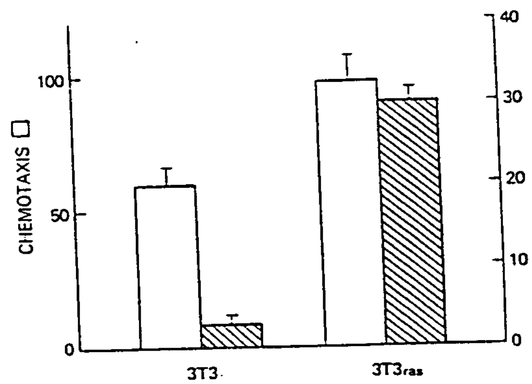


Fig. 3. Comparison between the migration (chemotaxis) and invasion activities of 3T3 and *ras*-transfected 3T3 cells. The cell migration assay was carried out as previously described (18).

Table 2 *Comparison of in vitro invasiveness and in vivo metastatic ability of primary and established prostate cell lines*

| | No. of cells which have invaded/field | No. of animals developing visceral metastasis after intrasplenic injection/total animals |
|---|---|---|
| Benign prostate hyperplasia | 0 ± 0 | 0/10 |
| DU 145 (prostate carcinoma line) | 2 ± 1 | 1/25 |
| Du LM (Du 145, liver metastasis) | 22 ± 6 | 9/10 |
| PC 3 | 17 ± 3 | 28/30 |

culture. The normal prostate and low-grade prostate carcinoma cells did not proliferate readily on the matrix, whereas the metastatic lines reached high density and totally lysed the matrix in the dish over the course of a few weeks (data not shown).

**Studies on H-*ras* Oncogene-transfected Cells.** *ras* oncogenes have been shown to be involved in a wide variety of human and animal malignancies (48, 49). The most frequent activating regions in these *ras* oncogenes have been localized to single point mutations at either position 12 or 61 in their coding sequences. NIH 3T3 cells transfected with *ras* oncogenes acquire a malignant phenotype and have been reported to show metastatic properties as well (50, 51). NIH 3T3 transfectants containing H-*ras* oncogenes with position 12 (T24/3T3) or 61 (Hs242/3T3) mutations were assessed for their invasive activity

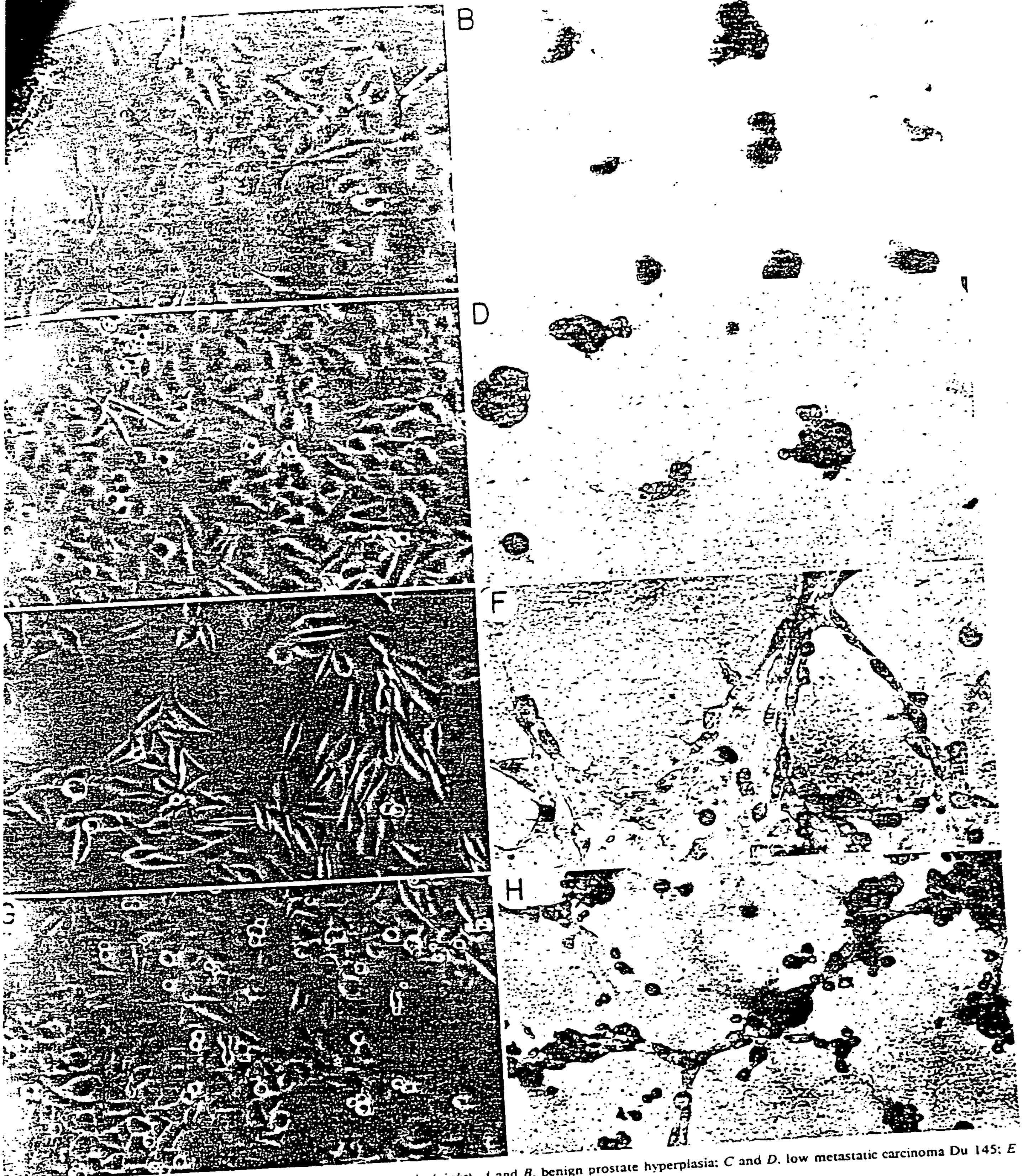


Fig. 4. Appearance of prostate cells on plastic (*left*) and on matrix (*right*). *A* and *B*, benign prostate hyperplasia; *C* and *D*, low metastatic carcinoma Du 145; *E* and *F*, high metastatic carcinoma (PC 3); *G* and *H*, high metastatic carcinoma line (Du LM).

*in vitro*. Both cell lines readily penetrated the matrix in the assay with little difference in their invasiveness (Fig. 2, *left*; Table 1). Both cell lines also formed branching colonies on matrigel-coated dishes (not shown).

## DISCUSSION

The invasiveness of tumor cells represents one of several important properties necessary for the formation of metastases (6). Many test systems have been used to obtain an assessment of the ability of cells to cross tissue barriers. Often whole organs or complex tissues such as the amnion, lens capsule, and bladder have been used (20), as well as *in vivo* determinations (51). It is assumed that it is the basement membrane in these tissues that creates a critical barrier to the passage of cells, although this has not yet been shown directly. Recently Terranova *et al.* (25) compressed collagen IV and laminin into a disc along with collagen I and interposed this barrier between two chambers, one of which contained tumor cells. Over the course of 1-2 days, cells from metastatic cell lines were found to migrate through the barrier, particularly in response to certain factors present in the tissue to which the cells appear to be targeted (19). Benign tumor cells and normal cells, with the exception of neutrophils, would not penetrate the disc. The barrier to the passage of cells was shown to be collagen type IV and laminin.

It was the purpose of the present study to develop a simple, more rapid method to assay the invasiveness of tumor cells through basement membrane. The 1 to 2 days required for the cells to migrate through the compressed layer of collagen IV, laminin, and collagen I necessitates sterility during the assay. Here we found that we could adjust the amount of matrigel on the filter and in this way vary the stringency of the barrier. Few cells from any source migrated through the reconstituted basement membrane at >100 $\mu$g/disc over the course of 6 h and all cells showed the ability to cross the barrier at lower levels of protein (12.5 $\mu$g/ml). An excellent degree of discrimination was observed at 50 $\mu$g/ml matrigel. At this concentration, the invasiveness of the cells was correlated with their malignant potential. In this assay, the cells cross the basement membrane matrix more rapidly than has been observed in other *in vitro* test systems using tissue matrices (1-3 days) (20) and *in vivo* systems (3-5 days) (52). These data are not inconsistent with our findings since the amount of time required for the cells to invade in this assay is directly proportional to the amount of matrix present. In addition, the *in vitro* assay described here does not have a continuous layer of endothelial cells on top of the basement membrane. It is estimated that the endothelial cell layer delays tumor cells from contacting the basement membrane *in vivo* for up to 2 days (52). The introduction of chemoattractants to the bottom chamber in the form of fibroblast-conditioned media speeded the movement of the invasive cells and was found to be necessary for maximal penetration over a 5- to 6-h period. This is in accordance with the suggestion (19, 20) that the migration and invasion of malignant cells into a target organ may be partially dependent on local chemotactic factors. Recently, autocrine motility factors have been described in tumor cell-conditioned media (53). The kinetics of the invasion process suggests that the numbers of cells crossing the barrier may increase with time as cells penetrate through the tunnels created by the first wave of migrating cells, as suggested previously by Kramer *et al.* (26), who also used matrigel as a substrate.

This rapid chemoinvasion assay measures the ability of the cells to (*a*) attach to the matrix; (*b*) degrade the matrix; and (*c*) migrate toward a chemoattractant. These events are considered to be important steps in the metastatic spread of tumor cells through basement membrane *in vivo* (20). Evidence for the degradation of the matrigel during the migration of the cells has been observed at the morphological level (26). Further, the addition of inhibitors of collagenase to the invasion chambers has been found to reduce the degradation of the matrix and to block cell invasion, and collagenase IV was found to be produced by the malignant cells and was detected in the lower compartment of the Boyden chamber.[5] The degradation of labeled collagen IV added to the matrigel was also observed.[5] These results indicate that invasion requires proteolysis of matrix components. While cell migration is a necessary activity for tumor cell invasion (13, 14), many nonmetastatic and highly metastatic cell lines give comparable chemotactic responses. Since in the chemoinvasion assay only the metastatic cells invade, it appears that the ability to degrade the basement membrane barrier is essential for the passage of these cells.

All cells characterized as invasive and metastatic *in vivo* which we tested were able to invade matrigel *in vitro*. NIH 3T3 cells were not invasive but NIH 3T3 cells transfected with activated *ras* oncogenes were able to cross the matrigel. In addition, MCF-7 breast carcinoma cells transfected with the *ras* oncogene were found to be highly invasive in this *in vitro* assay (54). These data are in accordance with other reports describing *ras*-transfected cells as being metastatic and invasive (50, 51). It is possible that this assay could be adapted and used to select for cells acquiring invasive properties after transformation with oncogenes or with DNA from metastatic cells.

Cells invading the reconstituted basement membrane can be isolated and studied. These cells appear to be a more invasive subpopulation of the parent line as determined on reassay. Such behavior is consistent with reports of Fidler and Kripke (55, 56) who showed that tumor cells are heterogeneous and that the invasive cells form a subpopulation in the parent tumor. We have preliminary evidence that some matrigel-selected cells are not only more invasive *in vitro* but are also more metastatic *in vivo*.[6] However, we do not expect that this will be true with all cells, since a variety of other factors including antigenicity could be limiting their spread.

Perhaps the most striking results were obtained with the prostate cells. Again invasiveness *in vitro* was correlated with the biological behavior *in vivo*. While the malignant and benign cells show little difference in morphology on plastic substrates, they are readily distinguished when grown on matrigel with the malignant cells forming many processes. Such differences in appearance and in activity as well as in the ability to isolate the more invasive cells in the population suggest that this material will be of general use in studying the interaction of cells with basement membrane. Such an assay also has the potential for being used as a rapid *in vitro* method for screening agents that inhibit invasive behavior, but are not neccesarily cytotoxic.

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SEMINAR

# Molecular Pathology of Tumor Metastasis III.

*Target array and combinatorial therapies*

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**Therapy of tumor progression and the metastatic disease is the biggest challenge of clinical oncology. Discovery of the diverse molecular pathways behind this complex disease outlined an approach to better treatment strategies. The development of combined cytotoxic treatment protocols has produced promising results but no breakthrough in the clinical management of metastatic disease. The multiple – specific and non-specific pathways and cellular targets of tumor progression are outlined in this review. Such an approach, individually designed for various cancer types, may have a better chance to treat or even cure cancer patients with progressive disease.** (Pathology Oncology Research Vol 9, No 1, 49–72, 2003)

*Keywords:* metastasis therapy, molecular target, cancer cell, host cell

### *Introduction*

Recently, advances in molecular cancer research have revealed numerous new therapeutic targets, some of which have already been tested in clinical settings. However, the clinical management of cancer patients is still based on surgical and/or radiological eradication of the primary solid tumor and chemical/radiological anti-proliferative/cytotoxic treatment of the disseminated disease. Despite a great deal of improvement in the control of loco-regional disease, only slow progress has been seen in systemic disease. As a result, mortality of cancer patients is still high due almost exclusively to the development of metastases. One obvious explanation for this discrepancy could be a difference between the molecular pathways controlling tumor growth and tumor progression (as analyzed in detail in the previous publications of this series).[1,2]

By definition, antimetastatic therapy covers all those available approaches which can prevent cancer cell dissemination or eradicate already disseminated and arrested tumor cells or their growing colonies outside the primary site, irrespective of the size of the cell population. Since metastatization (or tumor progression) is a complex phenomenon, its effective inhibition requires more than an anti-proliferative protocol.

Management of primary cancer and its eradication is primarily based on surgery and/or irradiation. On the other hand, chemotherapy is applied mostly as "anti-metastatic therapy", with the exception of the treatment of hematological malignancies and the primary chemotherapy of solid tumors. Since the process of metastasis is a complex interplay between the disseminating cancer cells and the host, rational and successful anti-metastatic interventions may target all the participants of these interactions in which anti-proliferative/cytotoxic interventions are only parts of a much more complex approach.

This review intends to describe all the possible targets in the metastatic cascade, which could be attacked pharmacologically, and to provide experimental and clinical examples for their therapeutic potentials. On the other hand, we will not review the currently available radio- and chemotherapeutic protocols (including endocrine therapy) of advanced cancer extensively documented in the current literatures.

### *Pathomechanism*

Since chemical compounds with pharmacologically exploitable properties may act on pathobiological events and interfere with one of the relevant molecular mechanisms it appears highly important to analyze the series of steps in

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the metastatic process and to attempt to select their appropriate inhibitors. It is conceivable that antimetastatic therapy might not be limited to a priviledged drug but hopefully will cover multiple agents acting on molecular mechanisms implicated in the sequence of the metastatic cascade.[1] Certainly these pathobiological events represent only those sequential steps that are strictly related to the passage of the tumor cells from the primary tumor to the secondary site. For the full completion of metastasis the tumor cells must have high viability to survive the circulation and have the potential for growth and vascularization. These latter events however occur in the primary lesion as well and their biological consequences do not necessarily lead to metastasis. There are overlaps in the molecular mechanisms in the metastatic cascade (i.e. shedding and proteolysis, which occur at several steps), in addition, some features may be present in both the primary tumor and the metastatic lesions.

There are two unique features in the metastatic process that have come to light recently, namely its inefficiency (i.e., only a small proportion of the tumor cells escaped from the primary tumor can form metastatic lesions) and the transition of the carcinoma cells between the epithelial and mesenchymal pheno- or genotype.[3] Until now, it was assumed that most tumor cells entering circulation are killed by monocytes, lymphocytes or leukocytes. However, in experimental models it was shown that a great proportion of tumor cells can survive the hostile circumstances of the circulation and their viability and growth potential are controlled by the secondary site. This implies that in hematogenous dissemination the initial steps (intravasation and survival in the circulation) could be much more efficient processes than the subsequent ones (extravasation and survival at the secondary site).[4] Consequently, the fate of disseminated tumor cells is determined by the new environment which controls growth to allow persistence and the formation of micro- and macroscopic lesions, or death of the tumor cells.

Another principal question concerns the uniformity in the mechanism of tumor dissemination, which must be considered seriously when designing antimetastatic therapy. The prevailing model of metastasis holds that few tumor cells with metastatic potential (one in ten million) within the large primary lesions acquire metastatic capacity through somatic mutations.[5] The genotypic heterogeneity of the tumors characterized by identical histology indicates the existence of diverse mechanisms involved in the progression of the individual tumors. Therefore it came as a surprise that metastatic solid tumors of diverse histological types have been found to have a common gene expression profile.[6] Since in lung-, breast-, prostate adenocarcinomas and medulloblastoma (but not in lymphoma) a similar tendency of association between gene expression profiles and metastasis has been observed, a generic gene expression program related to metastasis rather than distinct mechanisms of metastasis in different tumor types has been proposed. The characteristic set of genes expressed are designated as a molecular signature of metastasis, and show a close relationship with overall survival of the patients. Perhaps more important is the finding that this gene expression profile can be identified in a subset of primary tumors with higher metastatic capacity, suggesting that propensity to metastasize is a consequence of the predominant genetic state of the primary tumor and making less plausible the emergence of rare cells with metastatic phenotype in large tumors. These results argue for the development of metastatic phenotype during malignant transformation and indicate that the metastatic potential may be encoded in the entire tumor, consequently providing guidelines to identify tumors with high propensity to metastasize before surgery. Nevertheless, it must be mentioned that the above gene expression profile associated with metastasis includes many less characterized genes not listed among those known to contribute to the invasive/metastatic potential of tumor cells. Rather, these genes are expressed by stromal components of the tumor tissue, supporting the important contribution of the host to the entire process.[7] There is a vast amount of clinical data indicating that the incidence of metastasis is not simply related to the size of the primary tumors. Micrometastasis can appear in patients with small, low stage tumors and also in the absence of clinically detectable primary tumors.[8] Thus the chronic and systemic features of malignant disease indicate the importance of planned patient control after surgery and the availability of an arsenal of pharmaceutical interventions for the prevention/treatment of disease progression.

Below, we will summarize all the possible pharmacological targets and approaches developed to date, which are tested at least in experimental metastasis models or in the clinic for the treatment of advanced metastatic disease. These approaches can be divided into metastasis-specific modalities targeting those molecular events that are specific for the metastatic cascade, and nonspecific modalities targeting the primary and the metastatic tumor tissue as well as the dissemination process itself. Furthermore, pharmacological approaches are also divided according to their targets, i.e. tumor cells or host cells (tumor-host interactions).

## Specific anti-metastatic therapy

### Target: tumor cells

#### *Membrane receptors*

##### *Integrins*

Tumor cell-extracellular matrix (ECM) interactions are key events of various repetitive steps in the metastatic cascade involving ECM recognition, concentration of proteases to the invadopodia and migration on matrix sheets.[7] In various cancer types a well-defined integrin receptor

pattern develops, which promotes the emergence of the invasive/metastatic subpopulation. In special circumstances individual integrins can even become predominant over other matrix receptors offering at least a marker of these invasive cells.[1] Once a predominant integrin is identified on the surface of invasive cancer cells it offers a target for therapeutical intervention.[9]

Since most ECM proteins share the consensus sequence, RGD, for integrin binding, ***small peptide inhibitors*** of invasiveness and metastasis have been primary options. *In vitro* data provided ample of evidences that RGD-ligand peptides are powerful inhibitors of cancer cell adhesion, migration on matrices and in experimental metastasis models.[10] Such peptides showed also anti-invasive, anti-metastatic activity, especially in the case of hematogenous dissemination.[11] Unfortunately none of these peptides have yet entered clinical testing. Recently, following the same concept, larger ***RGD-containing peptides of fibronectin with fibril-forming potential*** have been designed and tested in experimetal models of various human cancers.[12] On the other hand, natural RGD-containing inhibitors, called ***disintegrins*** have also been identified in the venoms of various snake species. These natural peptide inhibitors of integrins are powerful inhibitors of cancer cell ahesion to various matrices including basement membrane as well as of cell migration, and are active in vivo in experimental metastasis models, especially in the case of hematogenous dissemination.[13-16]

Neutralizing/inhibitory antibodies against integrin receptors have pharmacological potentials too. However, unlike anti-growth factor antibodies, only a few such antibodies have been applied as anti-cancer agents. ***Vitaxin*** is an $\alpha v\beta 3$ integrin inhibitor humanized antibody which was primarily designed as angiogenesis inhibitor.[17] However, several cancer types are characterized by prominent $\alpha v\beta 3$ integrin expression and in these cases this integrin has been shown to be involved in the complex process of invasion and metastasis.[1] Meanwhile, to date there are no experimental data available concerning the direct anti-metastatic potential of Vitaxin. Another humanized anti-integrin antibody, ***abciximab*** (ReoPro) was introduced to clinic as anti-thrombotic agent targeting the platelet integrin $\alpha IIb\beta_3$ and the endothelial $\alpha v\beta 3$.[18] Abciximab not only inhibits platelet aggregation with or without the presence of tumor cells but also angiogenesis.[19] Ectopic expression of the $\alpha IIb\beta_3$ integrin has been observed in various cancer types including melanoma (also known to express $\alpha v\beta 3$).[20] Using experimental human melanoma metastasis models, abciximab has demonstrated anti-tumoral and anti-metastatic effects, encouraging further investigation of this subject.[19]

There is another rationale to target surface integrins on invasive/metastatic cancer cells. *In vitro* data have indicated that matrix adhesion induces drug resistance through overexpression of certain integrins. Such a phenomenon can be observed both in the case of hematological malignancies as well as in solid tumors.[21] An anti-integrin approach to the treatment of metastatic tumors may have the long-awaited „side effect" of reducing the drug-resistance of cancer cells.

### *Growth factor (GF) inhibition*

One of the earliest pharmacological agents discovered as a GF competitor was ***suramin***, a polysulfonated naftylurea.[22] The polyanionic molecule was able to sequester several growth factors with heparin binding domains including EGF, IGF, TGF-β, bFGF and PDGF. Several of these GFs are involved in the invasion and metastasis of various cancer types, and therefore it was expected that suramin would be an antimetastatic agent. However, suramin was too toxic and its non-specific binding to serum proteins limited its biological effect in vivo.[23] Accordingly, ***pentosan sulfate derivatives of distamycin A*** have been designed (heparinoids) and tested in experimental models. These compounds have anti-mitotic and anti-angiogenic effects and are able to compete with at least bFGF and IGF, suggesting that they could be developed as anti-metastatic agents too.

An alternative approach would be to use neutralizing anti-GF antibodies to suspend auto- or paracrine stimuli to cancer cell invasion. The feasibility of such approach is demonstrated in angiogenesis, where anti-VEGF antibodies have been demonstrated to be active ***in vitro and in vivo*** in preclinical models.[24] However, development of other anti-GF antibodies have not yet been reported in the literature.

### *Growth factor receptor (GFR) inhibition*

The majority of growth factors involved in the malignant phenotype of cancer are also motogenic factors: frequently, the same mitogen regulates the migration of cancer cells through partially degraded matrix. Accordingly, targeting the receptors for these GFs can provide an approach to target the regulation of cancer cell migration too. Cancer cells frequently overexpress GFRs at their surface due to genetic alterations in their genome, i.e. amplification. Most frequently such genes are members of the EGFR family.[25] EGFR2 (c-erbB2) is amplified and overexpressed in a subset of breast cancer characterized by a more agressive, more metastatic phenotype[26,27] but it has also been demonstrated to be expressed and functioning in ovarian- lung-, prostate- and GI-tract cancers (adenocarcinoma) as well as in head and neck cancers (squamous cell cancers).[25] EGFR1 (c-erbB1), on the other hand, is overexpressed mostly in squamous cell cancers, and in some adenocarcinomas too (such as colon cancer).[25] Another reason to target GFR on invasive cancer cells is that there is a cooperation between the signaling pathways of integrins and growth factors, and parallel inhibition of the two initiators of cell migration promises more

success in shutting down the motogenic activity crucial for cancer cells. Although there are several alternative options for targeting EGFR (specific ligand-mimetics, and *inhibitory antibodies*) only the latter approach has proved to be successful clinically. The best example is *Herceptin*, a humanized anti-c-erbB2 antibody, which is active clinically in advanced breast cancer overexpressing c-erbB2. The molecular consequences of the application of anti-c-erbB2 antibodies indicated that they downmodulate the surface receptor, thereby inhibiting the signaling cascade, but also initiate complement-mediated cytotoxicity as well as antigen-dependent cytotoxicity against their target.[26,27] Its success could well be followed by *IMC-C225*, an anti-c-erbB1 antibody that already has a humanized version. This antibody proved to be very effective against cancer progression in preclinical models.[28,29]

It is a novel approach to inhibit GFR expression at the level of transcription. The c-met oncogene and its ligand HGF have been demonstrated to be important regulators of the invasive/metastatic phenotype of various cancer types including colon cancer and melanoma.[1,30] Furthermore, this receptor system has also been implicated in the liver metastatic potential of various tumors thereby offering another rationale for anti-receptor anti-metastatic intervention. There are two tested pharmacological approaches in the literature, one targets the ligand, HGF, and the other the expression of the receptor.

The HGF ligand competition approach has used two strategies, a „splice variant" recombinant HGF with receptor-inhibitory potential[31] and the exploitation of the heparin-binding potential of the ligand and its importance in the conformational activation. Both the *inhibitory ligand peptide NK-4* (either recombinant or incorporated in adenovirus vector [31,32]), as well as a *peptidomimetic ligand of the heparin binding domain of HGF*[33] exhibited anti-invasive/anti-metastatic activity in preclinical models including effects on liver metastasis. Furthermore, since HGF is also an angiogenic factor, both approaches resulted in the inhibition of tumor-induced angiogenesis,[31,34] where NK-4 seemed to be more potent.

The approach to transcriptional regulation exploited the unique potential of *geldanamycin* (a member of the family of anisamycin antibiotics). This drug exhibited very selective inhibitory activity on the expression of c-met in cancer cells resulting in the down-regulation of c-met signaling and loss of the invasive/motile phenotype in experimental systems.[35]

### *Transmembrane proteoglycans*

Proteoglycans at the surface of cancer cells have been demonstrated to be involved in tumor progression in a Janus-faced manner.[36] In certain tumors downmodulation of their expression could be observed during carcinogenesis, while in other tumors overexpression occured when the tumor became invasive and metastatic.[36,37] The major players in this respect are the transmembrane type heparan sulfate proteoglycans (HSPGs: syndecans and CD44v3) and GPI anchored HSPGs of the glypican family. Their function is regulated by the glycanation process, which adds either heparan sulfate (HS) or chondroitin sulfate (CS) chains to the core protein. These proteoglycans, mostly through the HS-chains, are involved in cytokine/growth factor recruitment as well as in matrix adhesion processes, serving as co-receptors.[36] Furthermore, the transmembrane forms (syndecans and CD44) have been involved in important signaling processes such as vnt (syndecan-1), PKCα (syndecan-4) or motility signaling (CD44v3). These diverse functions of transmembrane HSPGs in the invasion process make them potential targets for therapeutic interventions.

It is well documented that in invasive/metastatic cancer cells glycanation of membrane proteoglycans is frequently shifted toward heparan sulfates from chondroitin sulfates offering a target for pharmacological interventions.[36,37] It was shown early on that using nonspecific glycosaminoglycan (GAG) biosynthesis inhibitors (β-xyloside, 2-deoxyglucose) it is not possible to alter invasive phenotype of cancer cells unless only the heparan sulfates are preferentially affected.[38] *5'hexyl-2'-deoxyuridine (HUdR)* preferentially inhibits HS biosynthesis in cancer cells.[39-41] Such treatment does not affect cell proliferation, but rather inhibits tumor cell-ECM interactions, thereby down-regulating the metastatic potential.

A unique approach for the inventional use of GAGs has been developed when neoglycans were prepared from albumin and CS or HS GAGs.[42] Among these new glycans neoCS demonstrated impressive anti-tumoral pro-apoptotic effects *in vitro* and *in vivo* in experimental breast cancer and myeloma. Further studies are required to explore the pharmacological potentials of these new GAG agents.

### *Cell adhesion molecules (CAM)*

Local invasion (previously defined as shedding), representing the first step in metastasis, may occur if the intercellular links and – in the case of the epithelial cells – firm contact with the basement membrane have been drastically altered. Subsequently the detached tumor cells are not confined by their neighbours and are ready for invasive growth. Assuming that more detailed knowledge of these cell contacts could be utilized in drug development, the question has been raised whether loss or gain is the dominant feature in the molecular mechanisms implicated in shedding.

At present cadherins, through the formation of adherent junctions, are regarded as the critical molecules both in homophylic and heterophylic intercellular contacts.[43] It is noteworthy that MDA-MB-231 human tumor cells, after transfection with E-cadherin cDNA, lose their capacity to form osteolytic metastases.[44]

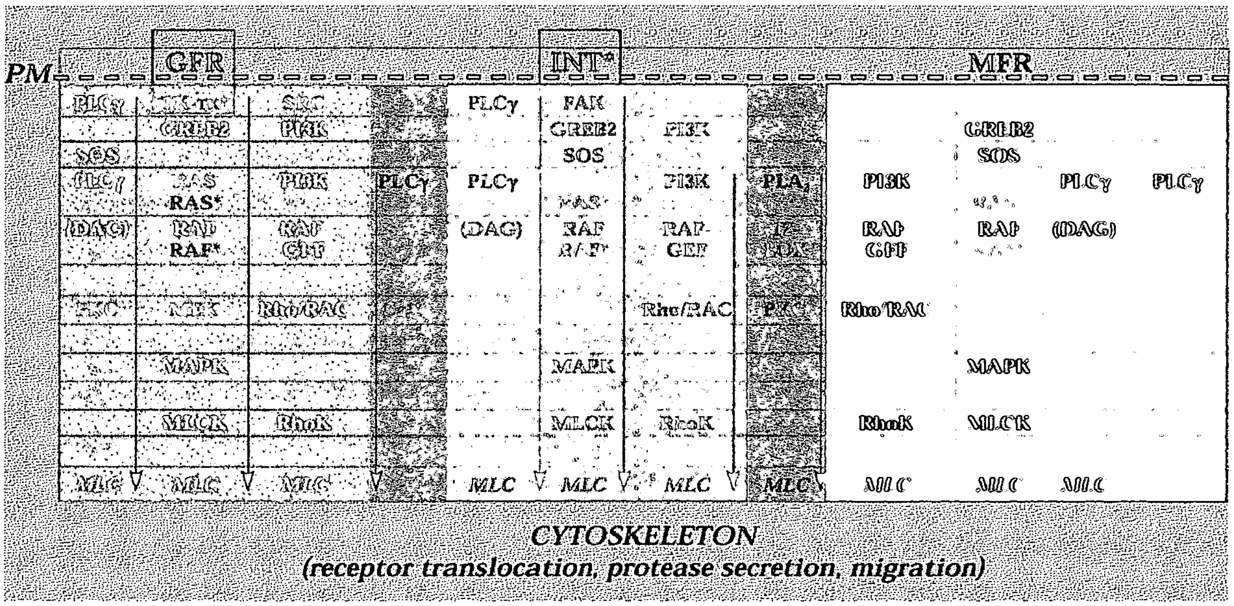



*Figure 1. Signaling pathways in tumor cell invasion. GFR: growth factor receptor; INT: integrin receptor, MFR: motility factor receptor; MLC: myosin light chain; PM: plasma membrane; TK: tyrosine kinase; INT*/TK*/RAS*/RAF*: activating mutations. Red: pharmacologically targeted signal elements*

Several clinical studies have concluded that E-cadherin expression is reduced in various malignant tumors (prostate, stomach, kidney, head and neck, etc.). This correlates with the metastatic potential of the tumors, therefore cadherins are considered as invasion/metastasis suppressor molecules.[44] Based on these observations it was suggested that administering agents that up-regulate E-cadherin expression may be a potential therapeutic approach to inhibit tumor spread.

Defects in E-cadherin functions can occur despite high levels of expression by steric hindrance of cell surface proteoglycans.[45] In this latter case experimental reduction of the glycanation of cell associated proteoglycans with β-D-xyloside resulted in the abrogation of tumor cell invasiveness without influencing E-cadherin expression.

### *Signal transduction*

Tumor cell invasiveness and metastatic potential depend critically on the signaling events generated through the interactions with ECM and the auto-paracrine regulatory factors of motogenicity *(Figure 1)*. These signaling pathways are highly similar to those functioning in normal cells as far as their final downstream targets are concerned, but the upstream pathways are highly diverse in the various types of cancer depending on the specific genetic background (such as RAS mutations, oncogenic splice variants, deletions, fusions etc). Furthermore, these signaling pathways are differently controlled at receptor level since both the expression of adhesion receptors and cytokine receptors can be altered in cancer cells. Accordingly, signal transduction inhibitors could be potentially useful in controlling invasiveness and the metastatic potential of cancer cells. This idea is further corroborated by the recent findings on metastasis suppressor genes where a subset of these genes controls those signaling pathways that are critical for metastasis and does not affect the mitotic signaling, arguing for the existence of invasion-specific signaling pathways.[46]

### *Receptor tyrosine kinase (PTK) inhibitors*

Although several PTK inhibitors have been recently developed to target the tyrosine kinase activity of EGFR,[47] VEGFR[48] and ABL/c-kit/PDGFR,[49] their effects on the regulation of tumor cell motility is largely unknown. Today several small molecular EGFR inhibitors are in *clinical trials*[50] and some of them have already demonstrated activity against head and neck-, ovarian- and non-small cell lung cancers.[50,51] It is interesting that c-ABL is one of the many downstream kinases of the integrin-linked signaling,[52] which already has a clinically successful inhibitor (*Gleevec*, imatinib mesylate, in chronic myloid leukemia[49]). Future studies will reveal if this drug has any anti-invasive potential in solid tumors. On the other hand,

there are a series of PTKs that could well be anti-invasive drug targets, including the integrin-associated FAK, c-met and c-src.[52] A small molecular inhibitor of src (PP2) has been shown to be active *in vitro* in various cancer types and even inhibited liver metastasis formation in a preclinical model, offering a promising pharmacological alternative.[53]

*G proteins*

Autocrine motility of tumor cells is regulated by two ectoenzymes, AMF/phosphoglucose isomerase and ATX/ecto-phosphodiesterase/lysophospholipase D.[54,55] While the receptor of the former was identified as a chemokine-type receptor,[56] it is still unkown in the case of the latter. The most upstream element of both signaling pathways contains *pertussis toxin (PTX)* sensitive G proteins.[57] PTX was shown to be a highly potent inhibitor of cancer cell migration *in vitro* and *in vivo* in experimental models and was tested recently in *clinical trials* againts bladder cancer as a local treatment. [58] On the other hand, a clinically available anti-thrombotic agent, *cilostazol*, targeting the phosphodiesterase activity of ATX has shown a potent anti-motile effect *in vitro* on cancer cells, suggesting that this autocrine loop could be specifically targeted in cancer. [59]

*RAS*

Downstream elements of the two signaling systems (adhesion and migration) involves RAS, which is a target of extensive investigations.[51,52] Since active RAS is anchored to the lipid bilayer by farnesyl transferase, *inhibitors (FTI)* of this process have been designed. [50,51] Although these inhibitors showed great promise initially in preclinical models, they turned out to be *clinically inactive*. A major problem with FTIs is that there is an alternative mechanism for RAS anchoring (geranylation) and the inhibitors act preferentially on H-RAS while in human tumors K-ras and N-ras are predominant. The antisense approach is also being tested clinically where both H-RAS and its downstream partner RAF-1 are used as target (results are yet unknown).[60-62] However, recently it was reported that activating BRAF mutations (but not RAF-1) are frequent in certain human tumor types, suggesting BRAF as a potential target in the RAS- (and motility-) signaling.[63] *BAY43-9006 is a non-selective RAF inhibitor*, which could well be the future drug for human tumors with BRAF activations and it is now in *clinical trials*.[51]

*MAPK*

A major downstream signaling target of the integrins is MAPK, involved in both matrix interactions and cancer cell motility.[50,52] Around one third of human cancers shows activation of the RAF-MAPK system, suggesting it as a feasible anti-invasive target. MAPKs (1,2) are phosphorylated by dual specificity kinases MEK1,2 and selective non-ATP-competitive antagonists already exist (PD98059 and U0126, see for review in reference).[51] These inhibitors affect cancer cell proliferation, survival and migration *in vitro* and *in vivo* in experimental models. Furthermore, *orally active variants of MEK inhibitors* have been developed and are now being tested in clinical trials.[51]

*Lipid signaling*

Activation MAPK in integrin signaling can be achieved through various pathways. A classical one is mediated through FAK/SOS/RAS pathway but alternatives exist and one of them is lipid signaling.[52] In this case integrin ligation or constitutive activity stimulates phospholipases-Cγ, D or $A_2$. Activated PLCγ pathway will result in PKC activation while $PLA_2$ activity will lead to the activation of the arachidonic acid pathway (COXs or LOXs).[52,64] Experimental data demonstrated that certain integrin signaling pathways involve the activation of 12-lipoxygenase providing bioactive lipid (12-S-HETE) for activation of PKC [65] as well as of cyclins.[66] Furthermore, the involvement of 12-LOX-PKC in the motility signaling of AMF was also documented.[67,68] Accordingly, both matrix adhesion as well as cancer cell migration dependent on the activity of 12-LOX enzyme of cancer cells.[64] Pharmacological inhibition of tumor cell 12-LOX inhibited tumor cell – matrix interactions (adhesion, protein degradation and migration) *in vitro* and metastasis formation *in vivo* in experimental models,[69,70] suggesting *lipoxygenase inhibitors* as feasible anti-metastatic agents.

COX-2 was demonstrated to be involved in GI tract carcinogenesis as well as in angiogenesis,[71] although the exact role for this enzyme in signaling is not known yet. Analysis of the autocrine signaling by AMF in cancer cells revealed the involvement of COX enzymes upstream of the target tyrosine kinases but downstream of the G proteins.[68] Recent studies on human colorectal cancer cell lines indicated that the *pharmacological inhibition of COX-2* by etodolac or JTE-522 results in the inhibiton of migration and secretion of MMP-2 *in vitro* and inhibition of liver metastasis in preclinical models.[72,73] These data indicate that at least in colorectal cancer COX-2 inhibition could be developed to form anti-metastatic regime specifically targeting tumor cells.

*PKC*

PKCs have been shown to be involved in the mitogenic and motogenic pathways, including integrin signaling and have been considered feasible drug targets for a long time. However, PKC is a family of serine/threonine kinases with variable structure and function. PKCs involved in cytoskeletal functions (adhesion and motility) are the, iso-

forms: these are therefore to be considered as anti-metastatic drug target. Several PKC inhibitors have been developed but few have yet *entered clinical trials*. The best known inhibitor is *bryostatin* which has been shown to be active *in vitro* and *in vivo* in preclinical models, though its clinical activity is minimal.[74,75] On the other hand, *seleno-compounds* exhibited chemopreventive potential targeting PKC (at least the Ca-dependent isoforms) and resulting in sustained inactivation.[76] Although these molecules have shown promise in various carcinogenesis models they have not been tested in experimental metastasis models. On the other hand, it is a great challenge to know how these inhibitors would be able to discriminate between PKC in normal cells and those in the cancer cells since mutations or major structural alterations of these enzymes are not known.

### *$Ca^{++}$ signaling*

$Ca^{++}$ is another important secondary messenger in the signaling pathways,[77] but in special circumstances it could well have a primary (signal initiator) function too. Classically, PLC activation in various signaling pathways leads to the generation of IP3, activation of the intracellular $Ca^{++}$ stores and an inrease in intracellular calcium level. However, activation of certain membrane receptors can also directly induce influx of $Ca^{++}$ into the cells through Ca-channels (voltage or ligand gated forms) and/or also liberate intracellular $Ca^{++}$ from the intracellular stores (as a kind of third messenger function). Major intracellular downstream targets of $Ca^{++}$ are Ca-dependent protein kinases (ie. PKC), but several EF-hand proteins should also be considered (calmodulin, calpain, Ca-dependent ATP-ase, aequorin etc.). Delineation of the molecular details of the RAS signaling identified that it is significantly modulated by intracellular $Ca^{++}$, suggesting another form of cross-talk between various signaling pathways.[78] The involvement of Ca-signaling in tumor cell adhesion-spreading-migration sequence was recognized early on, identifying it as a potential target for pharmacological interventions.[79] Experimental data already indicated an anti-metastatic potential of *classical Ca-channel blockers* of all classes (phenylalkylamines, benzothiazepins and dihydropyridine).[80] Treatment of cancer cells with Ca-channel inhibitors modulates integrins at the plasma membrane, rearranges cytoskeletal filaments and inhibits tumor cell-platelet interactions,[81] all critical events in dissemination.

*Carboxyamido-triazol (CAI)* was identified as a new pharmacological inhibitor of $Ca^{++}$-influx in the cancer cells, inhibiting the generation of secondary messengers, protein tyrosine phosphorylation and even gene transcription.[82] CAI was defined as a classical anti-metastatic agent inhibiting invasiveness *in vitro* and *in vivo* in experimental models. Furthermore, CAI was also identified as an anti-angiogenic agent.[79] CAI was and is being tested in *Phase I trials* in refractory solid tumors[83] and has shown frequent stabilization of the disease. In another setting CAI was used in combination with taxane in relapsed ovarian cancers showing some activity.[84] Collectively, these data suggest that $Ca^{++}$ signaling in cancer cells can be considered as anti-metastatic target and further studies are warranted to identify clinical relevance of this approach.

## Target: tumor cell-host interactions

### *Proteases and protease inhibitors*

The fundamental role of matrix-degrading enzymes in cancer invasion and metastasis was recognized following the pioneer work of Liotta and their collegues back the 80s.[7] Since then the biological role of the three main protease classes (matrix metalloproteases: MMP, plasminogen activators and cathepsins) has become more and more complex. Initially MMPs were considered to have pure protein degrading function with their main targets in the ECM around the invasive cells, while later it turned out that they can be linked to the cell surface (some members are even transmembrane proteins), and can bind surface receptors such as integrins, CD44 or transmembrane HSPGs.[85] They are not only present at the cell surface of tumor cells but they also regulate proliferation and apoptosis. Studies also revealed that normal cells are equal contributors to the protease repertoire of invasive cancers. Major attention was attracted when the role of MMPs was revealed in tumor-induced neo-angiogenesis.[24] Pathological studies defined the protease patterns of major cancer types and identified uPA as the most common protease expressed and active in human cancer.[1] Meanwhile the expression of MMPs (MMP-2/9 – gelatinases, MMP-3/10 – stromelysin, MMP-7 – matrilysin, MMP-14 – MT-MMP) and cathepsin B and D in various cancers in association with progression was documented (colorectal-, breast- or prostate carcinoma[85,86]). Accumulating experimental data on the role of the natural protease inhibitors in the down-modulation of metastatic potential suggested proteases as the first molecular targets for metastasis-specific therapies.[86]

Since at molecular level MMPs were well-characterized, design of MMP inhibitors started early on and the experimental data supported the rationale that inhibition of MMP activity modulates the metastatic capacity of malignant cells. There are two major classes of MMP inhibitors, targeting the synthesis or the activity of the enzymes.[85] Inhibition of the biosynthesis of MMPs can be achieved by blocking their transcription by antisense technology,[87,88] using *ribozyme* (MMP-7/9),[85,86] or *downmodulating the signaling pathways* controlling it.[51] More recently *halofuginone* was identified as a fungal inhibitor of MMP expression and was shown to be biologically active in

experimental metastasis models.[89] The MMP activity inhibitors are usually small molecular inhibitors, although initially the use of the natural inhibitors was suggested, this concept did not prove useful. The three classes of MMP activity-inhibitors are *collagen peptido- and non-peptidomimetics and the tetracyclin analogues*.[86] The peptidomimetics are competitors for the cleavage site of the substrate. These include *Batimastat* and the new variant, *Marimastat* (both form British Biotech), both having been tested in phase III *clinical trials*.[85,86] Marimastat showed some promise in advanced pancreatic and gastric cancers by inhibiting progression of the disease as compared to conventional chemotherapies.[85,86] Small molecular inhibitors designed for the active site of the enzyme have also entered clinical testing, *Prinomastat* (Agouron/Pfizer) and *Tanomastat* (BAY). Among the experimentally active tetracyclin analogues *Metastat* (Col-3) has entered clinical testing in Kaposi's sarcoma (combining the anti-tumoral and anti-angiogenic potential against a malignant endothelial cell tumor).[85,86] Some unconventional MMP inhibitors have also entered clinical testing, even phase III trials including *Neovastat* (the shark cartilage extract)[90] and the green tea component *EGCG (epigallocatechin-3-gallate)*.[91] MMPs can be used also to target conventional cytostatics (e.g. Melphalan) to the tumor tissue (*collagen peptide-cytostatic drug conjugate*) as it was demonstrated in experimental models.[92]

However, the trial data do not support the overwhelming enthusiasm that developed around the MMP inhibitors as new anti-cancer/anti-metastatic agents,[86] but the design of the trials and the selection of cancer types to test their effects was and still is most probably the major cause. These invasion-inhibitors were tested clinically in advanced cancers already at a stage where invasion and metastasis had developed and angiogenesis was initiated. Secondly, the tumor types selected as target were not those reported to overexpress MMPs at advanced stage and where MMPs are prognostic factors.[1] Considering these facts, the clinical activity of some of these inhibitors must be regarded as encouraging.

The cysteine-protease system involved in cancer invasion is the cathepsin family,[93,94] and small molecular inhibitors have been designed and tested in experimental models. Of major importance is the newly developed selective *cathepsin B inhibitor* CA-074 since several cancer types overexpress cathepsin B at their surface.[95] A less selective inhibitor family was designed to the nucleophylic thiol residues in the active site of the enzyme.[96] Although these inhibitors are active *in vitro*, they have not yet been tested in experimental metastasis models. Even less development is reported in the field of the serine protease pharmacology though the significance of uPA and uPAR system in invasion and metastasis of solid cancers is widely accepted.[1,97] This family of proteases is part of the fibrinolytic system, a potent activator of the MMPs and regulator of the integrin functions, suggesting it as an attractive multipurpose anti-invasive target.

### *Extracellular matrix*

Since the importance of a bidirectional relationship of tumor-host interactions in tumor progression has been widely accepted novel targeting of antimetastatic therapies toward molecular mediators of the tumor-host communication interface has been proposed.[7] Attachment of tumor cells to privileged host cells has been recently demonstrated showing that chemokine receptors expressed in tumor cells match the chemokines that are present in organs which are invaded by these tumor cells.[98] It is highly promising that blocking the relevant chemokine receptor can inhibit metastasis of breast cancer cells in experimental animals.[35,86] The fate of metastatic tumor cells depends on the interaction between the host cells and the tumor cells. Certain highly viable cancer cells may proliferate immediately after arrival into the new organ, sometimes even in the terminal capillaries before extravasation. More frequently, however, micrometastatic lesions are formed where the rate of apoptosis and cell proliferation is balanced.

The matrix milieu of the organs involved in cancer dissemination provides a strong selection factor for the entire process. Although this is evident from experimental data, the molecular details are not completely known, at least not to the point where clinical therapeutic modalities can be designed and tested. Bone metastasis provides an example how local ECM can influence metastasis formation and which kind of pharmacological targets could be used to be clinically successful in the selective treatment of metastasis.

Meticulous analysis of the pathomechanism of bone metastasis development revealed that osteoclasts are the key players in the bone resorption and that cancer cells are using them to initiate the process, while osteoblasts are also involved.[99,100] As a result, bone metastases are composed of lytic and plastic elements depending on the predominance of the key host cell types (osteoclasts and osteoblasts).

Data now indicate that active osteoclasts develop from a monocytic precursor upon the interaction with osteoblastic stromal cells. Key chemical mediators of this process are vitamin D3, PTH and PTHrP hormones (the common receptor is PTHR1), IL-6 and IL-11 cytokines as well as PGE2. Their molecular target is RANKL (member of the TNF ligand family) in the osteoblastic stromal cells.[100] This ligand activates its receptor RANK on mononuclear cells and initiate a cellular program of differentiation toward osteoclasts. This process is fine-tuned by a soluble TNF receptor, OPG, an inhibitor of the RANKL/RANK

interaction.[99,100,101] Bone-metastatic cancers are characterized by PTHrP expression,[2] believed to be responsible for the organ selectivity of the process and also for the priming of osteolysis (*Figure 2*). Bone matrix TGF-β further promotes the expression of cancer-PTHrP through a PKC-dependent transcriptional mechanism.[102-104]

The pathomechanism of osteoblastic bone metastasis is less well defined. It seems that endothelin-1 and its osteoblastic receptor, endothelin-A, are of primary importance in the activation process.[102] However, other mitogens such as IGF1, TGF-β or PDGF-BB may all be involved in various types of cancers metastatic to the bone. Certain proteases such as uPA and the serine protease PSA, have been reported to be involved in the development of osteoblastic metastases.

Therapeutic approaches to combat bone metastases are developing rapidly and have ***entered the clinical arena***.[105,106] This is due partially to the success of the development and widespread use of ***bisphosphonates***. These drugs targets the „soil" in the metastatic cascade of bone metastasis, covering the mineral components of the bone trabeculi, thereby inhibiting bone resorption by osteoclasts. Some experimental data, however, suggest that bisphosphonates have a direct anti-tumor effect as well.[100] Another target of bone metastasis is PTHrP produced by tumor cells. Its inhibition can be achieved by vitamin D analogues,[107] neutralizing antibodies[100] or even by new specific transcriptional inhibitors.[108] These approaches have shown conclusive results in experimental model systems and they are now under advanced clinical testing. The other molecular target is the RANKL/RANK system where the ***natural inhibitor OPG***,[109] and a ***chimeric ligand RANK-Fc***[110] have just entered clinical trials.

These approaches all target the lytic phase of bone metastasis, although osteoblastic mechanisms could be equally important. Unfortunately, only one therapeutic approach has been developed to date for this pathomechanism, and tested in experimental systems, an ***antagonist of endothelin-A receptor signaling***.[100] When future therapies are designed for the more effective management of the prevention and tretament of bone metastases, both osteoclastic and osteoblastic processes should be considered.

### *Circulating tumor cells and hemostasis*

#### *Coagulation*

Both circulating tumor cells and tumor tissues are in contact with the coagulation factors. In the latest case this is provided by the leaky new vessels produced during tumor-induced neoangiogenesis. Furthermore, it has been demonstrated in various cancer types that tumor cells can produce an array of pro- and anti-coagulation factors. However, cancer patients, especially those in advanced stages of the disease, are characterized by coagulation disorders, primarily a prothrombotic state.[111-114] Cancer cells produce tissue factor and/or cancer procoagulant, two major players responsible for this hypercoagulation.[112,113] Both factors activate the extrinsic coagulation pathway, cancer-TF must complexi with F-VII while cancer procoagulant directly activates F-X, ultimately leading to the production of thrombin. On the other hand, cancer cells can also express PAI-1,[112-113] which in turn promotes the expression of uPA, a physiological activator of the fibrinolytic system but also one of the most universal proteolytic enzyme of invasive cancer cells.[1]

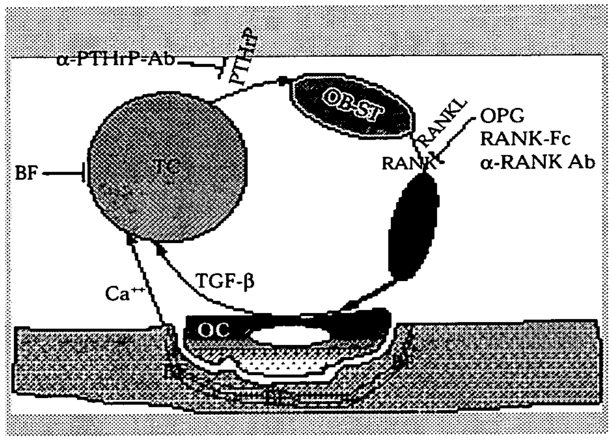



*Figure 2. Molecular targets in bone metastasis. BF: bisphosphonates, TC: tumor cell, OB-ST: osteoblast – stromal cell precursor, OPG: osteoprotegerin, Ab: antibody, OCP: osteoclast-progenitor, OC: osteoclast, PTHrP: parathormone-related protein*

Administration of ***anti-coagulant therapy*** to cancer patients revealed that such treatments not only eradicate thrombotic complications but also delay tumor progression.[115] However, such an anti-metastatic effect depends on the type of anti-coagulant applied. Agents that specifically inhibit thrombin, such as hirudin or warfarin, are less potent in this respect than the broad specificity heparin(s).[111,112,116] Recently ***LMW-heparins*** turned out to have interesting significant anti-metastatic effects in ***clinical settings***.[116,117] Analysis of the potential targets of heparin(s) in tumor progression revealed that tumor cell-platelet interactions (mediated by P-selectin), tumor-induced angiogenesis or tumor cell proliferation and migration are all affected besides the coagulation system.[118-120]

#### *Platelet-tumor cell interactions*

The involvement of platelets in tumor progression and metastasis is a three decade-old story in which the molecular mechanism(s) responsible for these processes were gradually revealed and refined.[114,122] Tumor cell-platelet aggregates defend tumor cells from mechanical damage as well as from nonspecific or specific attacks by neutrophils,

monocytes or NK cells. Both experimental data and now clinical studies indicate that platelets promote hematogenous metastasis. The mediators of this specific intercellular communication are surface adhesion molecules and β3 integrins in particular.[122] In the case of platelets the αIIbβ3 integrin is involved, while in the case of tumor cells both classes of the β3 family could be considered (αvβ3 or αIIbβ3).[123] Integrins of tumor cells and platelets are engaged and bridged by soluble RGD-containing matrix molecules of the circulation, vitronectin, fibrinogen and fibronectin. Importantly, during the specific adhesion of platelets to tumor cells in the vasculature both participants become activated, resulting in the production of various pro-invasive and mitogenic agents including growth factors, cytokines, bioactive lipids and proteolytic enzymes.[114] In most cases tumor cell-platelet interactions are complicated by the involvement of endothelial cells (during the intra- and extravasation phases of hematogenous dissemination). Beside the integrins, this complex interaction involves selectins and other cell adhesion molecules. It is another aspect of this story that platelets, especially when activated by tumor cells, initiate or promote angiogenesis.[114,122] In this way activated platelets indirectly promote the establishment of the metastatic tumor foci.

Based on our knowledge of the molecular players involved in this interaction several therapeutic modalities have been tested in experimental and even in clinical settings. One of the most promising agents is the anti-platelet antibody, ***abciximab*** (ReoPro) targeting platelet integrin αIIbβ3.[122,123] This antibody was shown the be ***clinically active*** in disrupting thrombi in myocardial infarction. Under experimental conditions abciximab inhibited hematogenous dissemination of murine and human tumor cells and tumor growth, partially by its inhibitory effect on tumor-induced angiogenesis.

Much attention has been given to the bioactive lipids involved in platelet aggregation. Since platelet-COX as well as the LOX enzimes are involved in platelet aggregation, their inhibitors have been tested primarily in experimental metastasis models with controversial results. A possible explanation for such an inconsistency is that platelets rely on these enzymes differently depending on the concentration of the agonist(s) (in this case tumor cells). At low agonist concentration tumor cell-platelet interaction activates the COX pathway exclusively, whereas at high agonist concentration the lipoxygenase pathway is also activated.[124] This implies that COX (especially TBX) inhibitors can have inhibitory potential only at low tumor cell/platelet ratios, whereas both COX and LOX inhibitors are necessary at a higher tumor cell concentrations. As a result, such inhibitors have been frequently reported to have no effect on experimental metastasis since they were used alone and not in combination.[124]

Prostacyclin, mostly produced in small amounts by platelets or endothelial cells, also has potent anti-platelet activity and was introduced early on as an experimental anti-metastatic agent.[114,122,125,126] However, further experimental studies on both PGI2 and their stable analogues indicated frequent failures of such treatments in inhibiting metastasis formation of various cancer types[125] – most probably due to similar problems as in the case of arachidonate metabolism inhibitors.

## NON-SPECIFIC THERAPEUTIC TARGETS OF TUMOR METASTASIS

### Target: tumor cells

#### *Modification of apoptotic response*

The inefficient apoptosis program is one of the most important factors in carcinogenesis. There are two main reasons for the lack of apoptotic response- both of which could be a target for therapy: *(a)* the apoptotic program is intact but it is inhibited by the continuous production of one (or more) survival factor(s); *(b)* the apoptotic program is damaged due to the underexpression of proapoptotic or overexpression of antiapoptotic signals. From therapeutic point of view, the activation of the apoptotic program either in the tumor cells or in those host cells which support tumor growth (e.g. endothelial cells in intra- or peritumoral vessels) could be an important contributor to therapy for both primary or metastatic tumors.

#### *Induction or increase of the activity of proapoptotic molecules*

The expression ***of death receptors and/or death ligands*** are key response elements to an outer apoptotic signal. Carcinogenesis (e.g. in the colon) can result in the loss of death receptor (e.g. Fas) expression.[127] It has been shown that chemotherapy can induce the expression of previously missing Fas in many neoplastic cells.[128] In cases when the lack of Fas receptor is the only deficiency along the apoptotic pathway, the responsiveness of tumor cells to FasL produced either by host cells (mainly lymphocytes)[129] or by neighbouring tumor cells (where the FasL production could also be induced by chemo- or any other therapy).[130] The latter effect may contribute to the „bystander effect" observed after gene therapy.

In colon cancer the ***Fas receptor*** can be induced by 5-fluorouracil through a p53-dependent pathway. [131] However, in many cancer types p53 is mutated or its pathway is damaged and it is not able to induce apoptosis. In that case agents as interferon-γ can „replace" p53 by activating Fas expression.[132] This is the reason why IFN-γ clinically supports the therapeutic effect of 5-fluorouracil.[133]

The ***clinical trials*** using the systemic administration of ***death ligands*** (TNF, FasL) have failed because of the tox-

icity of the ligands. [134,135] Nevertheless, there is a promising death ligand in oncology: *TRAIL*. TRAIL was found to be cytototoxic to many human tumor cell lines but did not cause significant toxicity in animal models.[136] The reason behind this selectivity against malignant cells is still unknown. The leading hypothesis is based on the observation that normal cells express more of the decoy receptors (DcR1 and DcrR2) than the tumor cells.[137] Nevertheless, treatment with TRAIL proved to be effective against glioma and colon cancer in preclinical models.[136,138] In breast cancer cells the effectiveness of TRAIL was dependent on the cytotoxic agent used in the combination. Doxorubicin or 5-fluorouracil - in combination with TRAIL – were synergistic, while methotrexate, melphalan or paclitaxel had no influence on TRAIL action.[139] The toxicity of TRAIL is still a question of debate,[140] but it is highly possible that the quality of the recombinant ligand from different sources is quite different.[141]

More and more agents are reported (lodinamine, arsenite, betulinic acid, CD437, and some amphipatic cationic α-helical peptides) which can *increase the permeability of the mitochondrial membrane*, acting either directly on the membrane or indirectly through the PTPC (permeability transition pore complex). This effect helps the escape of critical proapoptotic mitochondrial molecules (e.g. cytochrome c) into the cytoplasm and can induce apoptosis when other conventional anticancer agents are ineffective. *BCL2 family members* are important regulators of mitochondrial membrane permeability. In the case of decreased or missing expression of BAX or BCL-Xs the transfer of their genes into tumor cells could change the balance in favor of proapoptotic signals. In experimental models the introduction of an Ad-DF3-BAX destroyed 99% of tumor implants.[142]

*Caspases* could be reasonable targets to switch apoptosis on, however, it is very hard to ensure a selective toxicity against tumorous cells. In certain tumors (e.g. neuroblastoma, rhabdomyosarcoma, small cell lung cancer) the activity of caspase-8 is very low due to the hypermethylation of the promoter gene region. [143-146] There is hope that methylation inhibitors (e.g. 5-azadeoxicytidine) might restore caspase-8 expression.[147]

### *Inhibition of the activity of antiapoptotic molecules*

Most strategies aim at inhibition of either antiapoptotic molecules or the inhibitors of proapoptotic molecules. *FAP-1* can bind to FAS, preventing signal transduction from the receptor.[148,149] An oligopeptide has been synthesized and used against FAP-1 re-establishing FAS sensitivity.[148] *FLIP* has a much wider inhibitory action than FAP-1 on FASL or TRAIL induced apoptosis in experimental systems.[150] An antisense oligonucleotide against FLIP made cholangiocarcinoma cells sensitive again to FAS mediated apoptosis.[151] Such approach could be a useful component in a schedule based on TRAIL administration.

The overexpression of *BCL2* has been considered as prototypic reason for the inhibited apoptotic response. Since such gene errors due either to translocation or to amplification are present in many human tumors, the inhibition of BCL2 became a central challenge. An antisense oligonucleotide targeting the first six codons of the coding sequences has reached clinical trials *(G-3139, Genta)*.[152] It seems that this antisense therapy is more effective in combination with cytotoxic agents than given alone. In lymphomas antisense-BCL2 has been combined with cyclophosphamide, in small cell lung cancer with paclitaxel, in hormone resistant prostatic cancer with mitoxantrone, in breast cancer with docetaxel, in colon cancer with irinotecan, in relapsing acute leukemias with fludarabine and cytosin arabinoside, and in melanoma with dacarbazine.[152] Furthermore, *antisense oligoncleotides* have also been made against BCL-XL. [153]

Another inhibitory family is the *IAP* (including *survivin*). Antisense oligonucleotides have been applied in lung cancer and in melanoma with reasonable success. [154]

In many tumors the apoptotic response is inhibited by *survival signals*. In most cases such signals are suggested but not identified. One of the most active survival protein is AKT which is the effector molecule of PI3K pathway.[155] AKT is able to inhibit a variety of proapoptotic proteins. Any agent that inhibits AKT expression has a potential to revitalize these proapoptotic molecules. *PI3K* could be inhibited e.g. by wortmannin. The PI3K pathway is stimulated by many signals, e.g. ABL or RAS. Overexpression of *BCR-ABL* fusion gene or its product can be inhibited by an antisense-oligonucleotide or by a specific kinase inhibitor (Gleevec, imatinib mesylate, is now on the market for use in the therapy of CML and GIST etc.). [156]

## Target: tumor-host interactions

### *Tumor-induced angiogenesis and vascularization*

Vascularization of tumor tissue is an essential event in the establishment of both the primary and the secondary tumor lesions and can also be considered a key feature of the dissemination process (the metastatic cascade).[24,157] This is due to the fact that intratumoral blood vessels are the key structures of the intravasation phase of the metastatic cascade and their density directly correlates with the metastatic potential of several cancer types. In this way tumoral blood vessels are important targets of metastatic therapies. Since vascularization of the tumor tissue can be realized by various biological mechanisms various forms of targeting can be designed. This way, neoangiogenesis is only one among several pathomechanisms of tumor vascularization, but is almost the only field of anti-angiogenic pharmacology.

Based on the basic pathomechanism of tumor-induced angiogenesis a wide range of specific pharmacological inhibitors have been developed and even tested in clinical settings. Major classes of these inhibitors are anti-angiogenic cytokine inhibitors (targeting primarily VEGF), inhibitors of their receptors (primarily VEGFR) or the key integrin receptor, αvβ3, and the coupled signaling pathway (flk-1/kdr tyrosine kinase inhibitors) or specific endothelial cell inhibitors of various endothelial molecular targets (see in details in[24]). However, many molecular targets are shared by angiogenesis and tumor invasion such as MMPs,[85,86] cytokines (HGF, VEGF, IFN) and signal transduction pathways (EGFR[48]), so that pharmacological agents can have significant anti-angiogenic effects in addition to anti-tumoral and/or anti-metastatic one (see earlier). Furthermore, several classical cytostatic and cytotoxic agents used in clinics (not surprisingly) turned out to have anti-angiogenic effects as well, almost as a non-specific „side-effect" (*Table 1*). [24,157]

Clinical trials are now on the way to see if the enormous amount of experimental data can be turned to clinical benefit.[24,157] It seems that *IFN-α and Thalidomide* are two commercially available leading pioneers of anti-angiogenic agents reaching phase III trials for various advanced malignant diseases. [157] Among the experimental agents the natural anti-angiogenic factor *Neovastat*[90] and a tyrosine kinase inhibitor *SU-5416*[158] have also reached phase III trials. Accordingly, it is too early to predict or even outline the potential new anti-angiogenic modalities. On the other hand, it is important to mention that several classical cytostatic drugs regularly used today in various regimes have significant anti-angiogenic properties which could be better exploited (*Table 1*).

The key issue is to determine the precise place of anti-angiogenic therapy of the future. When an anti-metastatic effect is the surrogate marker and clinical goal, it is obvious that these new therapies have to be applied in an early phase of the metastatic cascade, immediately after elimination of the primary tumor showing at least local invasiveness or early signs of systemic spread (prevention of the vascularization of an established microscopic tumor tissue[159]). Such a treatment must be applied for an extended period of time, and potential side effects are therefore of major significance.[157] From this point of view natural agents or their recombinant variants with high specificity toward proliferating endothelial cells must have selective advantage. Another issue is that these new anti-angiogenic drugs are first tested clinically in phase I-II-III settings in advanced malignancies. In these trials anti-angiogenic agents are used to delay or inhibit new vessels in metastatic tumors which are already in an advanced stage of vascularization (well beyond the avascular size of 1-2 mm). Accordingly, frequent lack of anti-tumoral effects of these new agents is not a surprise and stable disease is as significant as a decrease of the tumor size.[157]

It is now accepted that in certain tumors cancer cells utilize the pre-existing vasculature of the host tissue and tumor vascularization actually requires remodeling of these vessels.[24] In this form of vascularization only those agents that can specifically target vessel remodeling can have a pharmacological role. On the other hand, other cancer types can redirect their genetic program and embryonic angiogenic geno- and phenotypes are developing resulting in the emergence of vascular mimicry of tumor cells and vascular channels made entirely or partially by tumor cells.[24]

Considering these options as well as the fact that already vascularized secondary tumors are frequently the clinical targets, the established tumor vasculature is considered as an anti-metastatic target. These anti-tumor vessel therapies may involve fundamentally different agents compared with classical angiogenesis inhibitors. Unlike the anti-angiogenic agents, unique molecular determinants of the tumor vasculature have outstanding significance for this therapeutic strategy. Such a molecular determinant could be VA-cadherin[160] where an inhibitory antibody toward this epitope on tumor-blood vessels could serve as an anti-vascular agent. Tissue factor can also be targeted to tumor vasculature by using a toxic conjugate, which than induces infarction of the tumor tissue in experimental models.[161] On the other hand, both VEGF/VEGFR as well as αvβ3 integrin on the tumor blood vessels can serve as targets and the anti-vascular effects of the VEGF-toxin fusion proteins,[162] anti-αvβ3 antibody (Vitaxin)[17] or cyclic RGD peptides[163,164] can be exploited to cause direct anti-tumoral/anti-metastatic effects and even improve radioimmunotherapy as it has been demonstrated in various preclinical models.

Collectively, the expanding knowledge of tumor-induced neoangiogenesis and the process of tumor vascularization now provide a vast array of molecular targets for specific therapy, but the rationale must be driven by our understanding of the various steps of the metastatic cas-

*Table 1.* **Classical cytostatic agents with anti-angiogenic potentials**

| | *In vitro* | *In vivo* |
|---|---|---|
| Cytotoxic for endothelial cells | Camptothecin/topotecan<br>Taxanes<br>Vinca alkaloids | Taxanes<br>Vinca alkaloids |
| Cytostatic for endothelial cells | Cisplatinum<br>Cyclophosphamide<br>Doxorubicin<br>Methotrexate | Cyclophosphamide |

cade. Only these considerations can lead to clinically successful anti-metastatic application of these new anti-angiogenic or anti-vessel modalities.

### *Tissue hypoxia and anemia*

Induction of neoangiogenesis in malignant tumors (both at the primary as well as at the secondary sites) is partly mediated by a hypoxia sensing mechanism.[165,166] The key molecular regulator of this system is HIF-1, a heterodimer of the HIF-1α transcription factor and ARNT/HIF-1β.[165] When $O_2$ is present HIF-1α binds VHL protein and the complex is ubiquitinated and degraded by the proteasome pathway. However, when $O_2$ is not present in the nucleus, HIF-1 can bind hypoxia-responsive elements (HREs) activating several target genes including pro-angiogenic ones like VEGF, bFGF, and other mitogens, pro-apoptotic genes, coagulation factors, genes involved in pH regulation or glycolysis, and even those involved in migration. However, a proportion of cancer cells is able to bypass hypoxic stress and develop a special phenotype involving radio- and chemo-resistance.[167,168] Importantly, the same tumor cell population is involved in the metastatic process, suggesting that hypoxia resistance could be an important factor that regulate the emergence of the metastatic phenotype. This is further corroborated by the fact that HIF-1α expression is increased in a wide variety of human cancers and serves as marker of poor prognosis.[166]

Anemia, the hallmark of the progression of various cancer types, develops on the basis of various pathomechanisms and results in general and local hypoxia.[169-172] Correction of anemia by rhEPO not only increased oxygen supply to normal tissues and improved quality of life of cancer patients but, surprisingly, improved response to radio- and chemotherapy, and even prolonged survival of patients.[168,173,174] However, regulation of hypoxia can serve as a double edged sword in cancer. Some aggressive tumors have a high oxygen consumption which generates hypoxia, but the HIF-1α pathway is abnormal and any correction of the $O_2$ level would simply further stimulate progression. In other tumors hypoxia in the tumor tissue generates the emergence of a more aggressive subpopulation through the involvement of the HIF-1α system. In this latter case correction of hypoxia will slow down the rate of generation of more aggressive cells (will turn off the hypoxic switch). Without the proper identification of function of the HIF-1 pathways in various cancers it will not be possible to identify those tumors where the correction of hypoxia can have an antimetastatic effect.[166]

### *Homeostasis: cancer cachexia*

About one fifth of cancer patients die due to cachexia, a severe loss of body weight from all the tissue compartments except the viscera. The majority of these patients are in an

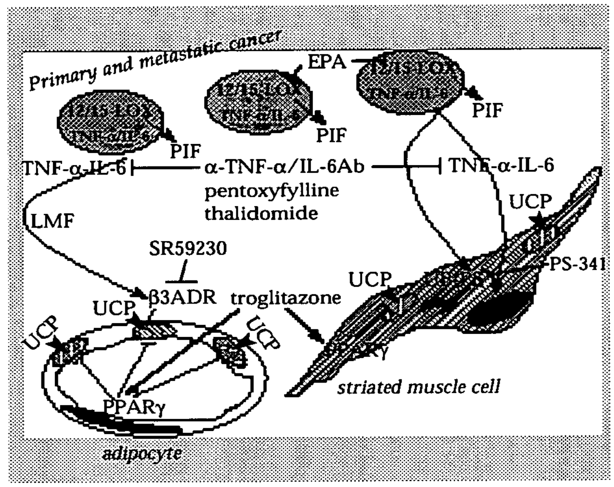



*Figure 3. Molecular pathways of cancer cachexia. PIF: proteolysis inducing factor, LMF: lipid mobilizing factor, UB-PS: ubiquitin-proteasome pathway, UCP = uncoupling protein*

advanced, metastatic stage of the disease. The loss of fat-free mass primarily involves muscle tissue. Since intracellular potassium is also lost, the process can be considered as bioenergetic deficit. However, unlike in starvation, liver mass is increased due to increased metabolic activities. On the other hand, cachexia not only characterizes the terminal stage of cancer progression but can also be present at an early stage of the disease and is a marker of poor prognosis which also affects response to therapy.

Cachexia in cancer patients is due to central loss of apetite, increased resting energy expenditure (REE) as well as to increased muscle protein breakdown/decreased synthesis and is regulated by different mediators (*Figure 3*).[175] Decreased apetite is considered to be induced by proinflammatory cytokines such as TNF-α, and IL-6, acting through the blockage of the hypothalamic NPY peptide. Interestingly, in experimental models cachexia was also mediated through MSH receptor, MC4R.

In certain cancer types, such as lung- or pancreatic carcinoma, REE is significantly increased contributing to the development of cachexia. This process is mediated by the abnormal function of mitochondria in the skeletal muscle.[175] Uncoupling proteins (UCPs) are responsible for balancing heat over ATP production. Tumor-derived TNFα as well as lipid mobilizing factor (LMF) increases the expression of UCPs in skeletal muscle and in adipose tissue in cachexia. The expression of UCPs is regulated by PPARγ,[176] suggesting a potential phamacological approach to compete with this effect of tumor tissue. Increased function of the Cori cycle in cancer patients is also responsible for increased REE.[175] This is primarily due to hypoxia and to the increased production of lactate by the tumor tissue.

Lactate is metabolized by the liver in the Cori cycle. In cachexic patients gluconeogenesis is attenuated too due to increased lipolysis (fat) and proteolysis (muscle). LMF-induced lipolysis was shown to be mediated through β3-adrenoreceptor.[175]

The progressive loss of muscle tissue is mediated through three complementary mechnisms, ubiquitin-dependent proteolysis,[177] TNF-α-induced downregulation of MyoD (a cell-type specific transcription factor)[178] and probably also by the activation of myostatin (a member of the TGF-β family).[179] The proteolysis of muscle tissue is induced both by TNF-α as well as by a sulfated glycoprotein, proteolyis inducing factor, PIF.[180] The latter acivates $PLA_2$ and lipoxygenases, ultimately leading to the production of 15-HETE.[175]

Based on this complex mechanism of cancer cachexia, several feasible pharmacological targets have been identified (*Figure 3*). As it was mentioned above, central loss of appetite is one (but not essential) target, where *corticosteroids or progestogens* have been clinically used as stimulators of NPY production.[175] On the other hand, the identification of the role of the MSH/MC4R system as regulator provides an alternative to develop inhibitory therapies.

The increased REE in cachexic cancer patients is another key factor in this complex mechanism. Systemic *anti-hypoxic interventions such as EPO administration* are obvious approach (see in details earlier). The nutritional deficit is the easiest target which can be reverted by protein- and energy-dense supplement but this approach alone is clinically insufficient. [175]

Ubiquitin-mediated protein degradation induced by TNF- and IL-6 provides a specific target for intervention.[177] Unfortunately, *anti-cytokine therapies* (mostly antibodies) have frequently been ineffective clinically.[175] On the other hand, *eicosapentaenoic acid, EPA* (and its natural source, *fish oil*) has been shown to specifically downmodulate the ubiquitin-proteasome pathway and proteolysis.[181,182] The effect is thought to be mediated through the inhibition of PIF production of cancer cells and downmodulation of lipoxygenase activities. The new inhibitors of the proteasome pathway (such as the *dipeptide-boronic acid analogue PS-341)* may have increasing role in this respect too.[183] To modulate protein turnover unbalanced in cachexia, *hydroxymethylbutyrate* as well as *arginine and glutamine administration* have been tested successfully in the clinic.[175] It has been demonstrated in preclinical models that the excessive lipolysis in cachexia induced by LMF and mediated by UCPs of mitochondria can be specifically targeted by β3 adrenoreceptor antagonists (such as *SR59230*[184]) or *PPAR activators* (such as troglitazone[176]).

Collectively it is now evident that cancer cachexia can be specifically targeted pharmacologically and even treated clinically (in a combined modality), providing new approaches for supportive care of tumor progression.

### *Immunotherapy*

The existence of tumor-associated antigens (TAA) and the detection of T lymphocytes recognizing these antigens, both systemically (in the peripheral blood) and locally (at tumor sites) in cancer patients, provides evidence that immune reaction can develop against metastatic cancer in these patients. However, the presence of tumor-specific cytotoxic T lymphocytes (CTL) most frequently does not translate into an effective antitumor immune response, as is reflected by the unarrested growth of tumors. Numerous mechanisms have been described that could contribute to the escape of tumor cells from immune recognition and destruction.[185] In order to boost immune responses against tumors, a variety of treatment modalities have been developed in preclinical and animal models, and tested in clinical setting. These include antigen-nonspecific approaches, as well as specific, active (stimulation of the host immune system) or passive/adoptive (transfer of effector cells or molecules) immunotherapeutical modalities. Some of these strategies were proved to be able to cause objective cancer regression, even of extensive metastatic disease, if only in a small percentage of patients, suggesting that a better understanding of the mechanisms of action of immunotherapeutical modalities may enhance the success rate of these strategies.

### *Nonspecific immunotherapy*

The application of nonspecific immunotherapy involves the administration of *bacterial immunostimulants* (most frequently *BCG*), as well as cytokines such as *interferons* or *interleukins*. Having been tested in a variety of malignant diseases, BCG remained accepted as a treatment of choice for the adjuvant therapy of superficial bladder cancer, probably representing the most effective immunotherapy in the case of solid tumors, based on a long-lasting local immune activation.[186] As an adjuvant, it is involved in many immunotherapeutical protocols involving different tumor types.

Among the cytokines, *interferon-α* (*IFN-α*) and *interleukin-2 (IL-2)* are most frequently used in immunotherapy trials. IFN-α has proved to be effective against a range of malignant diseases including, among others, melanoma, renal cell carcinoma (RCC), and Kaposi's sarcoma. In metastatic melanoma and RCC, IFN-α yielded 10-20% overall response rates as well as survival advantage,[187] while its benefit in the adjuvant setting of high-risk melanoma or RCC is more controversial. The mechanism of action of IFN-α is not exactly known, since beside immunomodulatory effects, it also has direct antitumor and antiangiogenic potential. IL-2 plays central role in immune regulation, primarily via its ability to stimulate the growth of T cells, but its effects involve the stimulation of NK cells, B cells and

macrophages as well. Similarly to IFN-α, most clinical experience has been gained in patients with metastatic melanoma and RCC, yielding approximately 10-20% overall response rates with complete response (CR) in 3-10% of the patients.[188] In patients achieving CR in response to high-dose IL-2 the regression of tumors at multiple metastatic sites was observed, and in most cases this response was durable. However, the mode of action of this regimen is not fully understood; although it was assumed that IL-2 exerts its antitumor effect via its stimulatory activity on T-cell proliferation and activation, Marincola and colleagues recently suggested that systemic IL-2 administration may facilitate T-cell function by promoting their migration and indirectly, through the activation of antigen presenting monocytes.[189]

*Active specific immunotherapy – tumor cell-based*

Numerous forms of active specific anti-tumor immunotherapy have been investigated in clinical trials in the past few decades, utilizing whole tumor cells or lysates, recombinant viral and bacterial vaccines, peptides, nucleic acid- or dendritic cell- (DC) based vaccines. Initial studies using *autologous tumor cells* (generally with BCG as adjuvant) resulted in moderate response rates. One of the disadvantages of autologous preparations, the potentially low amounts or weak TAAs, was attempted to be overcome using hapten-modified tumor cells with increased immunogenicity.[190] In these trials clinical response was generally associated with delayed-type hypersensitivity (DTH) skin reactions to autologous cancer cells. The most intriguing finding was that immunization with DNP-modified tumor cells induced inflammatory response consisting mainly of $CD8^+$ lymphocytes at metastatic sites in a significant proportion of patients.

Another approach to improve the efficacy of tumor cell vaccines is *transduction with genes encoding immunostimulatory cytokines*, which may enhance local immune response without causing systemic toxicity. The antitumor efficiency of these engineered tumor cell vaccines has been demonstrated in many animal studies. Clinical trials using *GM-CSF producing autologous tumor cell vaccines* in melanoma, RCC and prostate carcinoma patients[191,192] showed moderate clinical responses, and the development of DTH reaction to tumor cells, as well as a chronic inflammatory reaction in metastatic deposits in most patients, consisting of infiltrating T cells and plasma cells. Several forms of *allogeneic tumor cell vaccines* (whole cell-, lysate-, or shed antigen preparations) have also been tested in clinical trials, primarily in melanoma patients.[193] This type of vaccination was demonstrated to induce both cellular and humoral immune response in most patients, the extent of which was shown to be associated with clinical outcome. Survival data from phase II trials are promising, however, comparisons were made to historical controls only, and randomized phase III studies to confirm the therapeutic effect are either still ongoing or have demonstrated no statistically significant effects.

*Active specific immunotherapy – antigen-specific*

The identification of an increasing array of tumor-associated antigens and their respective HLA class I-restricted epitopes has opened new avenues for the antigen-specific immunotherapy of cancer. Of the potential forms of antigen delivery (recombinant viral or bacterial vectors, peptides, naked RNA or DNA, DC) the easy-to-produce, safe and reproducible *peptide vaccines* are the most studied. These peptides represent fragments of tumor antigens recognized by CTL in the context of a given HLA class I haplotype. In general, *peptide vaccines* (mostly administered without adjuvant or with weak adjuvants) induced the generation of cellular immune reaction against the peptide in about one half of the patients, but only limited clinical response rates, and there has often been no correlation between the immunological and clinical responses.[194,195] This dichotomy points to the inadequacy of currently used immunological assays for therapy monitoring and emphasizes the need for relevant intermediate endpoints measuring the activity of vaccines, which could predict clinical outcome.

Another potential problem arising from peptide vaccine trials is the selection of antigen-negative tumor cell populations due to down-regulation of the specific antigen and/or HLA molecule following immunization.[185,196,197] In theory, this could be overcome with the use of cocktails of peptides from different antigens. The application of whole antigen-vaccines (recombinant viral, bacterial, or naked DNA, etc.), including several epitopes presented by different HLA class I and class II alleles, would be available for a broader range of patients irrespective of their HLA haplotype, and would be able to target both $CD4^+$ and $CD8^+$ T cells. However, the documented effectiveness of these latter types of vaccines is low.[198,199]

Perhaps the most promising approach to improve antitumor immune response is the *application of DCs as vaccines.* These cells are the most potent APCs capable of eliciting strong antigen-specific CTL response in murine models and in humans. DCs from cancer patients can be cultured *ex vivo* and loaded with many different forms of tumor antigens: peptides, whole proteins, or cell lysates, followed by re-administration to the patients. Alternatively, DCs can be transduced with DNA or RNA encoding a given antigen or purified from tumor cells. Most clinical studies have been performed using *DCs pulsed with peptides or loaded with tumor cell lysates.* These trials revealed that such vaccines are able to generate immunity to tumor antigens without significant side effects, and objective clinical responses have been seen in some cases.[200-205]

An interesting approach involving immunization of RCC patients with *hybrids of autologous cancer cells and allogeneic DCs* was reported by Kugler et al.[205] Such a vaccine combines the high MHC class I and II expression and costimulatory molecules on DCs and the antigenic repertoire of the tumor cells, and was able to induce immunological response (DTH) against the tumor in 11 of 17 patients, as well as clinical responses in 7 patients.

### *Adoptive immunotherapy*

In addition to vaccination (active immunotherapy), the immune response against tumor antigens could be increased through the adoptive transfer of effector cells recognizing tumor antigens. Earlier attempts are exemplified by the administration of *in vitro* expanded *tumor infiltrating lymphocytes (TIL), in combination with high-dose IL-2* to sustain the proliferation of the infused T cells. This approach resulted in approximately 30-35% response rates in patients with metastatic melanoma and RCC, [206,207] however, in the case of RCC its benefit over IL-2 alone could not be confirmed in a randomized phase III study. [208] As a "revival" of this approach, Dudley et al. recently reported impressive results after *adoptive transfer of TIL-derived, in vitro expanded, highly selected tumor reactive T cells,* combined with high-dose IL-2, in metastatic melanoma patients conditioned by prior lymphodepletion. They achieved partial response in 6, and mixed response in 4 of 13 patients; in two responders clonal repopulation by tumor-reactive T cells persisted for several months.[209] In a parallel study, Yee et al. infused *PBMC-derived CD8+ clones* recognizing MAAs MART-1 or gp100, *in combination with IL-2,* resulting in accumulation of the transferred cells at metastatic sites, and a few minor or mixed responses, together with the selective loss of the targeted antigen in 3 of 5 cases studied. [210]

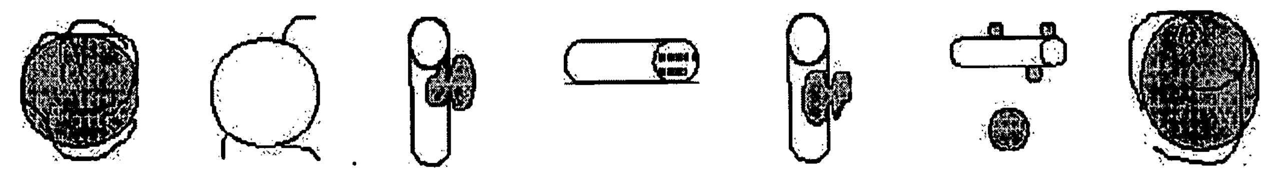
### *Comments to immunotherapy*

Several conclusions can be drawn from the results of published immunotherapy studies. First, these modalities are able to induce durable complete tumor regressions, even of extensive metastatic disease, mostly with reasonable toxicity; however, generally only in a minority of patients. This points to the importance of appropriate patient selection. In the case of antigen- or peptide-specific vaccination strategies it is important to examine the presence of antigens the relevant and HLA haplotypes at protein level, if possible, at all accessible tumor sites. Mixed responses, i.e., different responses of individual metastases are often encountered in immunotherapy trials, and probably reflect heterogeneity in the expression of tumor antigens, HLA class I molecules, apoptotic signals or immunosuppressive factors that might influence the effectiveness of an immune reaction. Furthermore, most tumor vaccines involve patients with advanced stage (or end-stage) disease, and evaluating the general immunocompetence of the patients prior to treatment has been done only in the minority of studies. A successful antitumor immune response may take several months to develop,[193] therefore patients with only 3-4 months life expectancy are less likely to benefit from these therapies. Although in some cases the regression of bulky tumors has been demonstrated in immunotherapy trials, it is possible that greater clinical impact could be obtained in the post-surgical adjuvant setting. Second, monitoring immunological responses as alternative study endpoints showed a lack of correlation with clinical outcome in many cases,[198,199,211] emphasizing the need for more adequate surrogate markers. There are promising newer tools requiring less *in vitro* manipulation that could prove useful in this respect, including assays detecting antigen-specific T-cell frequency (MHC-peptide tetramers), as well as functional assays detecting antigen-specific cytokine production by T cells (ELISPOT, cytokine flow cytometry, real-time quantitative RT-PCR).[212,213] The presence (even at high frequencies) of vaccine-elicited tumor-reactive CTL in the circulation, or in the tumors expressing the relevant antigens does not guarantee an efficient immune response leading to tumor regression. It is not clear at present if these T cells are in an activated and functional state, and, on the other hand, tumors can develop multiple mechanisms to escape immune recognition.[185] The molecules participating in these processes are not routinely tested in the tumors of patients enrolled in immunotherapy studies, nor monitored during the treatment. Finally, in several single target antigen-based clinical studies a therapy-induced immunoselection of antigen-negative clones has been observed in nonresponding tumor deposits, leading to disease progression.[185,196,197] This could be overcome by the use of antigen (peptide) cocktails or whole tumor approaches, either by themselves or as a DC vaccine. Moreover, therapies based on tumor antigens related to the process of malignant transformation or critical to the growth of cancer cells may be more resistant to immunoselection, and therefore be more optimal targets for immunotherapeutical interventions.[185]

### *Final comments*

At present metastatic tumors are principally treated with the currently available cytotoxic agents in clinical oncology which produce the most favourable responses. Alarmingly, there are experimental data suggesting the enhancement of metastatic potential after treatment with cytostatic drugs. Cylophosphamide was shown to enhance the formation of the metastatic nodules if the experimental animals were treated before the inoculation of the tumor cells. Unfortunately the elevated level of metastasis by cyclophosphamide could not be abolished by prostacyclin

*Table 2.* **Therapeutic approaches in the metastatic cascade. Target: metastatic tumor cell**

| | Metastatic Cascade | | | | | | |
|---|---|---|---|---|---|---|---|
| *Therapies* | *Primary tumor vascular, macroscopic* | *Local invasion ADM* | *Intra-vasation ADM* | *Circulation (Blood – lymph) A* | *Extra-vasation ADM* | *Dormancy / avascular micromets* | *Vascular macromets* |
| |  | | | | | | |
| **Cytotoxic (S/IR/CH)** | + | (+) | | | | | + |
| **„Anti-metastatic"** | | | | | | | |
| *Membrane receptors* | | | | | | | |
| Anti-GF/GFR | + | +(M) | +(M) | | +(M) | + | + |
| Anti-integrins | | + | + | + | + | + | |
| Anti-tm-HSPG | | + | + | + | + | + | |
| *Signal transduction* | | | | | | | |
| PTK-inhibitor | (+) | + | + | (+) | + | + | (+) |
| RAS-inhibitor (FTI) | | + | + | | + | + | |
| PKC-inhibitor | | + | + | | + | + | |
| Lipid signaling-inhibitor | | + | + | + | + | + | |
| Ca-signaling inhibitor | | + | + | | + | + | |
| *Survival/apoptosis* | + | + | | + | | + | + |

Abbreviations: A = adhesion (to matrix), D = degradation (of the matrix), M = migration (through the matrix), tm-HSPG = transmembrane heparan sulfate proteoglycan, GF/GFR = growth factor receptor, S = surgery, IR = irradiation, CH = chemotherapy

*Table 3.* **Anti-metastatic targets in tumor cell – host interactions (homeostasis)**

| *Therapies* | *ECM (host)* | *„Angiogenesis"* | | | *Hemostasis* | *Metabolism (host)* | *Immune defense* |
|---|---|---|---|---|---|---|---|
| | | *Hypoxia* | *Neoangiogenesis* | *Vascularization* | | | |
| **Protease inhibitors** | + | | + | | + | | |
| **ECM modifiers** (bisphosphonates) | + | | (+) | | | + | |
| **Angiogenesis inhibitors** | | | | | | | |
| Anti-hypoxic | | + | + | | | + | |
| Anti-angiogenic | | + | + | + | | | |
| Anti-tu-vessel | | | | + | | | |
| **Hemostasis regulators** | | | | | | | |
| Anti-platelets | (+) | | (+) | | + | | + |
| Heparins | (+) | | (+) | | + | | + |
| **Cachexia therapy** | | | | | | | |
| Apetite | | | | | | + | |
| Proteolysis | | | | | | + | |
| Lipolysis | | | | | | + | |
| **Immunotherapy** | | | | | | | + |

which, when administered alone, has a remarkable antimetastatic action.[214] Recently the development of metastatic capacity was observed among the survivors of MCF-7 human mammary adenocarcinoma cell population treated alternatively with FUdR and adriamycin.[121]

Furthermore, chronic and especially, low dose therapies with cytotoxic agents can also have unwanted negative side effects on tumor progression. The proposed metronomic scheduling of these therapies[215-218] does not consider the existing experimental and clinical data on this issue.[121,219-221] A more fundamental experimental approach is required to analyse the effects of these protracted therapies on tumor progression before testing them in clinical trials.

Identification of various molecular mechanisms, involved in the late phases of tumor progression leading to the development of invasive/metastatic capacity and metastatic disease, have already identified an array of new and specific targets for pharmacological interventions (*Table 2*). From the clinical standpoint major features of metastatic disease are all therapeutic targets (*Table 3*). Furthermore, it is now evident that expanding the survival of cancer patients with metastatic disease requires a more „holistic" approach to the disease where an array of targets have to be treated individually to reach the ultimate goal.

Similarly to cancer prevention, anti-metastatic therapies have the best chance to be active when applied in the early (perhaps subclinical) stage of tumor dissemination. Although this approach is widely tested in experimental models, there are no data to date on their clinical applicability. These interventions will require safe and selective agents which can be used for an extended periods measured in years (perhaps for the lifetime of cancer patients), and therefore the chronic toxicity of any agent in this field will be of outstanding importance.

The therapy of tumor progression and metastatic disease is the biggest challenge in clinical oncology. Pharmacological approaches using single agents which were standards early on in clinical oncology have changed considerably with the development of combined treatment modalities. These have produced promising results but few breakthroughs in the clinical management of the metastatic disease. The multiple pathways and cellular targets all have to be identified (molecular diagnostics) and included into combinatorial therapeutic regimes as outlined in this review where none of the individual components have a replaceable role. It is our expectation that such an approach individually designed for various cancer types may have a better chance to treat or even cure cancer patients with progressive disease.

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*Review*

# Drug Control of Solid Tumour Metastases: A Critical View

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**Abstract.** *Metastasis of solid tumours represent the target of election for the pharmacological treatment of cancer. Nevertheless, commonly used treatments do not represent any selective approach, provided that drugs are mostly unspecific cytotoxics. Today many strategies adopted to interfere with metastasis growth concern the interaction with biological signals of the metastatic cells or of the host. One difference should be made between anti-metastatic and anti-metastasis drugs, in that only the latter realise the goal of selectively destroying metastasis wherever they are. In this context many agents active on newly identified molecular targets are more effective in preventing metastasis formation than in inhibiting their growth. NAMI-A, an innovative ruthenium compound, seems to provide optimism for the future and, in laboratory models, it is very active on lung metastases independently of the stage of their growth. The success of NAMI-A against metastasis should stimulate laboratory studies with appropriate experimental models to predict clinical activity, since the use of experimental conditions closely similar to those of human tumours should help the identification of more active compounds.*



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## 1 Overview

*1.1 Solid tumor metastases.* Cancer is the second leading cause of death in most western countries. In the developed world, it affects around one in three individuals at some stage during their lives and causes one in five of all deaths. Cancer is not really a single disease, but a group of perhaps several hundred diseases. These all have in common the single factor that control over the growth of a particular bodily tissue, such as the lung, has been lost.

Although cancers are very heterogeneous, it is believed that they all have a similar cellular origin. They all display uncontrolled (but not necessarily very rapid) growth, which suggests that they are produced by malfunctions of the mechanisms which regulate growth. Another feature of malignant solid tumours is that cells may become detached from the main mass and can travel fairly freely around the body. If they become lodged in another tissue and begin grow there, which normal cells could not, they form the so called secondary tumours or metastasis.

Metastasis is the final frontier in cancer therapy. Given the importance of metastasis in cancer, the interference with this phenomenon represents a promising therapeutic strategy. The stage of tumour development at which metastasis occurs varies from one type to another. Tumours are often less cohesive than normal tissues which clearly contributes to metastasis. Tumours that metastasise early, while they are still small, have a poorer prognosis than tumours which do so when they are fairly large and easily detectable.

Almost half of all cancer deaths in the developed world are accounted for by tumour of the stomach, lung, breast, colon/rectum and cervix. These 5 tumours, as well as bladder cancer, oesophageal cancer, cancer of the prostate and skin cancer kill because they metastasise and at diagnosis they are disseminated and the complete removal of any but the smallest tumours is difficult. Therefore metastases of solid tumours represents the main reason of failure of cancer therapy. In fact, while surgery and/or radiotherapy have successfully cured the primary lesions, distant metastases bring invariably to death. Because of their almost ever scattered location, drug therapy appears to be the best choice. In fact, drugs have theoretically the

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possibility to distribute in the body and to reach each metastatic lesion in any possible location. However, in such an approach, today the weak ring of the chain is represented by the characteristics of the drugs available today.

*1.2 Cancer treatment.* There are four conventional primary methods to treat cancer: surgery, radiation, chemotherapy and immunotherapy.

Surgery cures cancers only if they usually are at an early stage and only in one place, *i.e.* localised. They must be in a site from which they can be removed with a good margin of normal tissue (because this may be infiltrated by invisible malignant cells).

Radiation is used when a region of the body is to be treated. Radiation inflicts injury and death upon the cells in its path, whether they are normal or cancerous. Usually normal cells are able to repair themselves better than cancer cells. Thus the cancer cells are selectively killed.

Chemotherapy, *i.e.* treatment by anticancer medicines given by mouth or by injection in the blood may reach all parts of the body and can treat cancer in any location. Almost all chemotherapeutic agents act by interfering with cell division. Normal cells which divide to replace those lost through wear and tear are also affected by chemotherapy.

Theoretically, immunotherapy represents the perfect treatment for cancer, because it could develop the body's own defences to kill tumours. Cancer cells are sufficiently different from normal cell so as to be recognised as a non-self and to provoke an immune response, but this does not happen in their host.

*1.3 Drug for metastasis treatment.* These drugs are the result of the so called "serendipitous discoveries", and are mainly characterised by the fact that they interact with cell division and growth by a cytotoxic mechanism often related to binding to DNA or DNA related mechanisms of cell division, they are not very tumour cell specific and have dose-limiting toxicity due to bone marrow and immune response impairment. Indeed, it should be stressed that the most important transversal link which is commonly shared by these drugs is the fact that they have been pre- clinically studied in *in vitro* systems or *in vivo* models with the principal aim to inhibit tumour growth independently of the test system adopted. Conversely, the last two decades have demonstrated the differences of solid tumour metastases and the primary tumours from which they arose, and particularly the different chemical sensitivity to the cytotoxic agents available compared to the primary counterparts (1-3).

No doubt these drugs still represent the only means for treating disseminated tumours, together with chemicals and other materials which induced recovery or activation of immune reactions against tumour cells as a result of rapid evaluation of the recently concluded trials available by a electronic search on Medline® and/or Cancerlit® reference system, under the key words tumour, metastases, human, treatment.

*1.4 Cytotoxic or ...* However, it should be clearly kept in mind that for many tumours, and particularly for breast cancer, "while cytotoxic or cytostatic drugs have found their place in treatment of disease, the death rate has not decreased" (4). Thus, there is a clear need for new therapeutic strategies for the control of solid tumour metastases. If on the one hand the use of the drugs available, should be continued by the optimisation of high dose and salvage treatments (5), the identification of new cytotoxic drugs endowed with novel mechanisms of action should also be developed as for example the new approach to immune-therapy by means of gene transfer and, more generally, genetic engineering of cancer or immune cells (6-11); on the other hand more emphasis should be put upon the translation of the enormous bulk of information coming from cellular and molecular biology of cancer cell into new treatment modalities (12-15). This new knowledge may perhaps allows us to overcome the systemic toxicity of drug treatment by the identification of new targets, specific for tumour cells, and therefore open a window of selectivity also for cancer chemotherapy, as suggested for breast cancer (16).

## 2 Molecular biology and metastasis treatment

The two hypothesis "selective toxicity" and "new molecular targets" may give rise to new treatment modalities. K. Hellmann wrote in 1987 "it is daunting to think that of the thousands of compounds which have been examined as potential cytotoxic anti-cancer agents, activity has been addressed only in terms of ability to prevent or interfere with cell division, as if this was the only malignant characteristic of tumour cells" (17). Greig and Trainer, almost at the same time, said that the "strategies to interfere with the paracrine signals that modulate metastasis growth can be directed at rendering the organ soil infertile and/or the tumour seed impotent" (13). Thus, 10 years ago, with less knowledge than today, people had already thought the idea of new drugs with new targets, perhaps with more specificity and therefore less toxicity. The way appears to be longer than expected/and none of the compounds under pre-clinical or even clinical development arose from any planned strategy (18-23).

Rather, it appears that, at least regarding the compounds under development at NCI, serendipity and casualty still direct the new discoveries. The result appears to be the acquirement of drugs with novel targets but with old and well known toxicity and dose-limiting effects (24).

*2.1 Anti-angiogenesis treatment.* A promising new target is represented by tumour angiogenesis. Under a purely

pharmacological approach, drugs directed to anti-angiogenesis therapies are represented by a) those capable of controlling the degradation of basement membrane, and b) those which interfere with growth factors implicated in the regulation mechanisms of signal transduction.

*It is now clear that many changes can be recognised on a cancer cell, such as the amplification of c-erb B2, c-myc, cyclin D1 and E, mutations of p53 and RB1 or down-regulation of nm23 and E-cadherin as shown in malignant cancers (25-32), or loss of cell-cell contact, altered interactions with biological substrates and of cytoskeleton which are associated to mutations of MSH2 and telomerase genes as found in colon cancers (33-35).*

Thus, considering that out of the many cells which escape primary tumour mass only a few realise form metastases because of the many unfavourable interactions with host environment which lead to apoptotic death (36-37), drugs which increase the possibility of these unfavourable contacts may considerably contribute to the death of metastasising cells, according to the 100- year old hypothesis put forward by Paget (38). With this hypothesis in mind, the success of treatment of experimental tumours with MMPs inhibitors [TIMPs (39) or Batimastat (40)], inhibitors of urokinase-type plasminogen activator and its receptor (41-43), drugs affecting PKC signalling pathways (44-45) and drugs affecting endothelial targets [TNP-470 (46)] should be investigated further.

In other words, these are treatments which may affect any single path of the complex mechanism of dis-regulation of the paracrine and autocrine signals of cell growth, and accordingly the metastasis process of solid tumours may be inhibited. The most impressive example might be represented by Batimastat (40) and Cicaprost (47).

*2.1.1 Batimastat.* Batimastat ([{4-N-hydroxyamino}-2R-isobutyl-3S-{thienyl}succinyl]-L-phenyl-alanine-N-methylamide, BB-94) is a compound with specific inhibitory activity on MMPs by a mechanism involving reversible binding of the zinc-binding region of MMPs with $IC_{50}$ ranging from 3 nM (cartilage collagenase) to 20 nM (stromyelysin), with no effect on the mRNAs of 72Kd type IV collagenase, matrilysin, stromyelysin and TIMP-2 (48).

✓ no tumour cell cytotoxicity

✓ significant reduction of number and weight of spontaneous lung metastases

✓ reduction of 72-kd and 92-kd collagenases activity

Batimastat controls the re-growth of breast cancer xenografts in nude mice and metastasis formation at doses of 30 mg/kg/day given for about 6 weeks. Batimastat did not reduce the number of mice with re-growth of the tumour after surgery and only marginally reduced that of the animals developing lung metastases but significantly and more markedly reduced the growth of the primary tumour to about 1/3 and that of lung metastases to about 1/2 of that of untreated controls (40).

*2.1.2 UPA inhibitors.* Cell-cell and cell-extracellular matrix contact and cell migration are also under the control of uPA, and such control has been proposed as a target for anti- metastasis activity for tumours in which uPA receptors have been demonstrated such as ovarian, colon and mammary cancers (41).

*2.1.3 Cicaprost.* The efficacy of Cicaprost on solid tumour metastases is based on another principle (49-51). Cicaprost is a metabolically stable prostacyclin analogue active after oral administration (47). Prostacyclin has been known since the eighties to interfere with certain steps of metastasis formation, and Cicaprost has pronounced anti-metastastatic effects in a series of spontaneously metastasising rodent tumours. The mechanism, at least referred to prostacyclin, should include the inhibition of tumour cell platelet aggregation, tumour cell adhesion and TPA and R-(S)-HETE- stimulated adhesion of tumour cells to endothelial cells, sub-endothelial cell matrix and fibronectin (50). With this mechanism of action, it is easy to explain the activity of Cicaprost on the prevention of metastasis formation. However, Cicaprost is also active on already established metastases. Such as those of tumours in advanced stage of growth, those residuing after surgery or those arising during surgical interventions. Thus, besides the inhibition of intra- and extra- vasation of tumour cells by inhibition of tumour cell migration and invasive capacity, Cicaprost also interferes with the adaptation of the metastasis cell in the micro-environment of the target organ by altering cell-cell contacts and tumour cell motility giving rise to growth inhibition mechanisms (52-55).

✓ no effects on primary tumour

✓ interference with several steps of metastasis process:

↓ intravasation;

↓ tumour cell-platelet interaction;

↓ tumour cell extravasation;

adaptation of metastatic tumour cell to target tissue microenvironment.

The activity of Cicaprost is shared by other agents with anti-thrombotic and anti-platelet activity such as many pirimido-pyrimidinic compounds, Rapenton (RA233), Nafazatrom and other inhibitors of cyclooxygenase and thromboxane synthase. However, this does not mean that anti-thrombotic is equal to anti- metastasis in that agents such as Bencyclin, Ditiazole and Ciproeptadine, strong anti-thrombotic agents, are devoid of anti-metastastatic effects (56).

*2.1.4 The cell signaling system.* Cell signalling targets and their inhibitors deserve a separate analysis and discussion, because of the many components involved, which is outside the present review; Focusing our attention on the PKC system and its various isotypes, it is interesting to note that it is possible to associate to the malignant phenotype specific characteristics not shared with the normal cells

from which it derived (57). Similarly, it is possible to observe the complete reversibility of such malignancy by *in vitro* treatment with specific agents capable of down- and up-regulating respectively those isotypes up- and down-expressed in the malignant cells. The actual bias is related to the impossibility to reproduce *in vivo* such results or to get results in the absence of side-effects. Bryostatin-1 and UCN-01, two examples of these compounds, show typical toxicity of cytostatic agents such as phlebitis [Bryostatin-1 (58)] and gastro-intestinal and bone marrow toxicity [UCN-01(59)1. Bryostatin-1 mimics the activity of phorbol, esthers on the PKC system, whereas UCN-01, an analogue of staurosporin, has a specific inhibition of PKC isoenzymes α, β and γ with less effect on PKC σ and ε and no activity on PKC ζ (60).

### 3 The "true" anti-metastatic drugs

The term anti-metastatic agent should be addressed only to those compounds which have repeatedly shown the capacity to selectively interfere with metastasis formation with marginal or no effect on primary tumour growth. With this restriction, it is necessary to discriminate the pure anti-metastatic agents from those whose activity is in part or totally due to reduction of primary tumour growth, the number of compounds remaining is reduced to three classes: a) Razoxane, b) Dimethyltriazenes and c) Ruthenium complexes. These compounds have a common denominator in the total absence of direct cytotoxic effects for tumour cells (property shared with Cicaprost and Batimastat), in the repeated evidence of anti-metastatic effects at doses devoid of activity on the primary tumour from which metastasis arose and in the capacity to interfere with spontaneous better than with artificially induced metastases.

*3.1 Razoxane* There is no doubt about the inclusion of Razoxane (1,5-dioxopiperazin-1-yl-propane) among the parental anti-metastatic agents. Moreover, Razoxane should be considered the first anti-metastatic compound for which the mechanism of the anti-metastatic action was proposed. Razoxane creates a wall around tumour blood vessels thus rendering tumour cell escape and penetration into blood stream less possible (61).

✓ inhibition of tumour cell dissemination:
  ↑ wall around tumour blood vessels
✓ toxicity against bone marrow:
  ↑ drug-induced leukaemias

Razoxane was also the first anti-metastatic drug for which a clinical trial was properly designed (62). "One concept for anti-metastatic treatment is that the drug must be present in the body for long periods in that its presence is necessary to prevent metastasis formation. Therefore anti-metastatic drugs have to be given continuously after tumour diagnosis and in this period they must be well tolerated and devoid of effects other than the prevention of metastasis formation (K. Hellmann)". Patients with colon cancer at Duke's A-C stages were treated for up to 60 weeks with 125 mg/kg/day and those with highest risk of liver metastasis (Duke's C) had a significant benefit from treatment in terms of reduced risk of development (18% vs 34%) and of metastasis free survival from tumour diagnosis (80 weeks *vs* 40 weeks) (63). Unfortunately, such treatment caused bone marrow toxicity and drug-induced leukaemia similarly to conventional cytotoxic drugs and these effects practically concluded the story of this anti-metastatic drug.

*3.2 Aryldimethyltriazenes.* Concerning the pure and selective anti-metastastic agents, compounds active in preventing metastasis formation, perhaps dimethyltriazenes represent the best example available (64). Conceptually derived from Dacarbazine (65), aryldimethyltriazenes include the best properties of a selective anti-metastastic drug: they inhibit the spontaneous formation of lung metastases of solid metastasising tumours and are virtually inactive on primary tumour growth, on the growth of already established metastases and on artificially induced (i.v. injected) lung metastases (66-68); they are also active on brain metastases of leukaemias (69). The mechanism by which these effects are exerted is complex and includes changes at primary tumour level with selection of cell populations with lower metastastic ability (68,70).

✓ no effects on primary tumour
✓ selective action against spontaneous metastases
✓ no effects on experimental or already estahlished metastases
✓ induce epigenetic alteration on tumour cells that stimulate host immunereaction

One aspect that would have rendered these drugs unique among the anti- tumour agents was that they induce an epigenetic alteration on tumour cells that gives rise to a marked increase of cell antigenicity and correspondigly to immunereaction against these transformed tumour cells and also their parental line (chemical xenogenisation), leading to the possibility to study a kind of vaccination against the tumour (71-73). The lack of attention to drugs of synthesis on the one hand, and the fact that the best results were obtained with compounds whose industrial development was not covered by appropriate patents are the main reasons for the discontinued studies on these promising drugs.

*3.3 Ruthenium sulfoxide complexes.* Ruthenium complexes characterised by sulfoxide ligands and ruthenium at +3 oxidation state are probably the most recent group of compounds with repeatedly reported anti-metastatic properties. One of them, *trans*-tetrachloroimidazoledimethylsulfoxide ruthenate (*trans*-$RuCl_4$(DMSO) Im] Na, NAMI), might represent a suitable model compound for anti-metastatic activity (74). The selectivity of the

effects on metastases in models of tumours spontaneously metastasising to the lungs is shown by the lack of effects on primary tumour growth at doses causing a remarkable reduction of lung metastases (75,76). On these metastases, NAMI is much more active on their weight than on their number, independently of the tumour being treated indicating that there is an effect on their growth than on their establishment. This statement is further supported by the observation that NAMI is effective on metastases not only by impeding their formation (treatment on early stage growing tumours) but also by reducing the growth of those already formed (treatment of advanced stage growing tumours) as shown by experiments in which the survival time of tumour bearing mice is taken as an end point of evaluation of the anti-metastasis effect (77-79).

✓ scarcely effective on primary tumour and on experimental metastases

✓ selective action against spontaneous metastases, also when already formed

✓ modulation of the balance MMPs TIMPs

✓ inhibition of tumour cell dissemination: ↑ wall around tumour blood vessels

✓ weak toxicity against host tissue at doses active on metastases

Besides the strong effect on metastasis responsible for a significant increase of survival time, ruthenium complexes such as NAMI or the more recent NAMI-A, for which a number of new observations will be available in this (80) and the next years, appear interesting because of the supposed mechanism of action. They modulate the balance between matrix metalloproteinases and their inhibitors by a direct action of the respective mRNAs and they increase the connective tissue matrix around blood vessels, thus impeding tumour cell escape, and around tumour mass (81). Correspondingly, although very similar to cisplatin because of the common belonging to an heavy metal structure and of a rapid biological half-life time with prevalent renal excretion (G. Sava, data on file), NAMI and NAMI-A have poor host toxicity at doses active on tumour metastases in mice (77,82).

## 4 Conclusions

Although surgical techniques and adjuvant therapies have reached a remarkable level of development, a large proportion of patients inevitably suffer for the formation of metastases from primary lesions. Eradication of metastases in patients with malignant tumours is thus the very important goal in clinical oncology. The actual panel of anti-tumour drugs, mostly discovered by chance or by semi-empirical procedure, has largely proved its inefficacy for treating disseminate disease and the need for anti-metastasis drugs is urgent. To achieve this goal for the development of a strategy for the anti-metastasis drug, the 100-year old Paget's theory of "seed" and "soil" still offers a conceptual useful tool.

The strategies adopted to interfere with the signals that modulate metastasis growth may be directed to render the "soil" inhospitable or the "seed" metastasis cell impotent. The process of haematogenous metastasis includes a sequential series of events: proliferation of primary tumour, vascular neogenesis, vascular invasion, transport into the blood stream, arrest in the capillary circulation of target organs, adhesion of tumour cells, extravasation, implant in the microenvironment and metastasis growth. It is thus necessary to define which step of the metastasis pathogenesis represents a serious risk for the patient and is therefore eligible for pharmacological intervention.

Anti-metastasis drugs must be considered separately from anti-metastastic drugs, in that only the former realise the goal of selectively destroying metastasis wherever they are. On the one hand, pharmacological approaches are represented by drugs discovered by chance, such as razoxane, with its neo-angiogenic effect, triazenes, which modify tumour phenotype, and ruthenium complexes with their effects on tumour matrix and proteases. On the other hand, they follow defined and aimed studies, such as that of anticoagulant therapy, to counteract some early steps of metastasis formation, or the immune-stimulant therapy, depending on the fashion: non-specific, specific, with toxins conjugated to antibodies, by the new strategies of gene transfer, or finally to the anti-invasive and anti-angiogenic therapies, mainly based on the inhibition of tumour matrix proteases and of tumour blood supply.

There is reason for optimism in the development of pharmacological approaches able to destroy the proliferation of tumour metastases. The development of the techniques of cell and molecular biology and of molecular genetics gives an important opportunity to address the perceptions of cancer biologists of the last decade.

On the one hand it is hard to discuss experimental models predictive for clinical activity, and on the other it should be stressed that some experimental situations are much closer than others to those usually encountered with humans. Similarly, clinical studies on novel therapeutic modalities often occur in unfavourable conditions on patients depressed by tumour growth and treatments that have significantly, if not totally, altered their physiological immune reactivity. It is not be surprising that these new therapies may show weak activity in contrast with the expectations derived from the preclinical examination.

One crucial point thus arises about the nature of the test systems to be used for the development of new drugs or therapies. An exhaustive work has been recently done by Welch (83) on this point, and there is no need for further discussion. However, since we focused on the pharmacological treatment of metastases it has to be stressed that many experimental conditions adopted to

claim anti-metastasis activity are of low or null value, where at least the use of artificially generated metastases and the use of tumours growing in immune-deficient animals represent two situations in which pronounced alterations of the biological interactions between host and metastasis are dramatically modified.

Finally, few words on the status of anti-angiogenic agents. The possibility of starving out tumours and preventing tumour cell detachement from the primary tumour and consequent metastasis formation is of great psychological impact also among scientists. However, our actual knowledge does not allow the identification of agents that selectively act on tumour cells alone. The obvious consequence is that "anti-angiogenic effects" are observed throughout the body and long term treatments, such as those required for antimetastatic therapy, will cause significant invalidating toxicities.

The hope of the authors of this review is that the criticisms raised throughout this excursus will open a debate on the strategies adopted and forecasted for the treatment of metastases. We again stress the importance of studying models predictive of the complex biological environment that drugs will encounter in humans, and we believe that this strategy will provide more reliable results. The statement that the most impressive drugs were found by chance rather than on a rational basis does not reduce the importance of planning new drugs on selective targets. Rather it means, in the opinion of the authors of this review, that the contribution of more experts of chemistry and pharmacology, to co-operate with molecular biologists and geneticists, is needed. We believe that this will be made possible at least by increasing the weight of studies on new anticancer drugs, besides that already put on the knowledge of the biology (genetics) of cancer cells.

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• REVIEW •

# Mechanism and its regulation of tumor-induced angiogenesis

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## Abstract

Tumor angiogenesis is the proliferation of a network of blood vessels that penetrates into cancerous growths, supplying nutrients and oxygen and removing waste products. The process of angiogenesis plays an important role in many physiological and pathological conditions. Solid tumors depend on angiogenesis for growth and metastasis in a hostile environment. In the prevascular phase, the tumor is rarely larger than 2 to 3 mm$^3$ and may contain a million or more cells. Up to this size, tumor cells can obtain the necessary oxygen and nutrient supplies required for growth and survival by simple passive diffusion. The properties of tumors to release and induce several angiogenic and anti-angiogenic factors which play crucial roles in regulating endothelial cell (EC) proliferation, migration, apoptosis or survival, cell-cell and cell-matrix adhesion through different intracellular signaling are thought to be the essential mechanisms during tumor-induced angiogenesis. Tumor angiogenesis actually starts with tumor cells releasing molecules that send signals to surrounding normal host tissue. This signaling activates certain genes in the host tissue that, in turn, make proteins to encourage growth of new blood vessels. In this review, we focus the mechanisms of tumor-induced angiogenesis, with an emphasis on the regulatory role of several angiogenic and anti-angiogenic agents during the angiogenic process in tumors. Advances in understanding the mechanisms of tumor angiogenesis have led to the development of several most effective anti-angiogenic and anti-metastatic therapeutic agents and also have provided several techniques for the regulation of cancer's angiogenic switch. The suggestion is made that standard cytotoxic chemotherapy and angiogenesis inhibitors used in combination may produce complementary therapeutic benefits in the treatment of cancer.

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## MECHANISM OF ANGIOGENESIS

Angiogenesis is a complex multi-step process involving extensive interplay between cells, soluble factors, and extracellular matrix (ECM) components. Four distinct sequential steps in angiogenesis include: (1) degradation of basement membrane by proteases; (2) migration of endothelial cells (ECs) into the interstitial space and sprouting; (3) ECs proliferation at the migrating tip; (4) lumen formation, generation of new basement membrane with the recruitment of pericyte, formation of anastomoses and finally blood flow[1]. The angiogenic response in the microvasculature is associated with changes in cellular adhesive interactions between adjacent ECs, pericytes and surrounding ECM. In the process of active neovascularization, activated ECs reorganize their cytoskeleton, express cell surface adhesion molecules such as integrins and selectins, secrete proteolytic enzymes, and remodel their adjacent ECM. These events are followed by the formation of capillary buds. Autocrine and/or paracrine angiogenic factors must be present to induce EC migration, proliferation, elongation, orientation and differentiation leading to the re-establishment of the basement membrane, lumen formation and anastomosis with other new or pre-existing vessels, eventually leading to the formation of intact microvessels.

## CANCER'S ANGIOGENIC SWITCH

Angiogenic phenotype serves the development of malignant tumor at multiple stages. Tumor cells may overexpress one or more of the positive regulators of angiogenesis, may mobilize an angiogenic protein from the ECM, may recruit host cells such as macrophages (which produce their own angiogenic proteins), or may engage in a combination of these processes. Tumor angiogenesis is mediated by tumor-secreted angiogenic growth factors that interact with their surface receptors expressed on ECs. The most commonly found angiogenic growth factors such as VEGF and bFGF, when encounter ECs, they bind to the tyrosine kinase receptors on ECs membrane. Binding leads to dimerization of the receptors and activation of autophosphorylation of tyrosines on the receptor surface and thereby initiates the several signaling proteins (including PI3-kinase, Src, Grb2/m-SOS-1 (a nucleotide exchange factor for Ras) and signal transducers and activators of transcriptions (STATs) each of which contains src-homology-2 (SH-2) domains[2]. Binding of the SH-2 regions of these proteins to the phosphotyrosines on the receptor tyrosine kinases (RTKs) activates several pathways that are crucial for triggering the cell cycle machinery. The most well studied pathway passes through the GTP-binding protein Ras and activates the mitogen activated protein kinase (MAPK) cascade and subsequently transcription factors in the nucleus[2]. Up-regulation of an angiogenic factor is not sufficient in itself for a tumor cell to become angiogenic, however, certain negative regulators or inhibitors of vessel growth may need to be down-regulated[3]. If there is a preponderance of angiogenic factors in the local milieu, the neovasculature may persist as capillaries, or differentiate into mature venules or arterioles. If instead, the local milieu changes such that there is a preponderance of angiostatic factors, the neovessels can regress. The angiostatic factors that mediate regression can do so either by inducing apoptosis or cell cycle arrest of ECs. Thus, the switch to the angiogenic phenotype is regulated by a change in the local equilibrium between positive and negative regulators of the growth of microvessels[1,3].

## FACTORS INVOLVED IN TUMOR ANGIOGENESIS

### *Vascular endothelial growth factor and receptors*

Vascular endothelial growth factor (VEGF), also known as

vascular permeability factor (VPF), is a heparin-binding angiogenic growth factor, and is highly expressed in various types of tumors. It may increase ECs permeability by enhancing the activity of vesicular-vacuolar organelles, clustered vesicles in ECs lining small vessels that facilitate transport of metabolites between luminal and abluminal plasma membranes[4]. Alternatively, VEGF may enhance permeability through mitogen-activated protein (MAP) kinase signal transduction cascade by loosening adhering junctions between ECs in a monolayer via rearrangement of cadherin/catenin complexes[5,6]. In addition, recent studies have shown that VEGF enhances ECs permeability by activating PKB/Akt, endothelial nitric-oxide synthase (eNOS), and MAP kinase dependent pathways using human umbilical vein endothelial cell[7] (Figure 1). Increased vascular permeability may allow the extravasation of plasma proteins and formation of ECM favorable to endothelial and stromal cell migration.

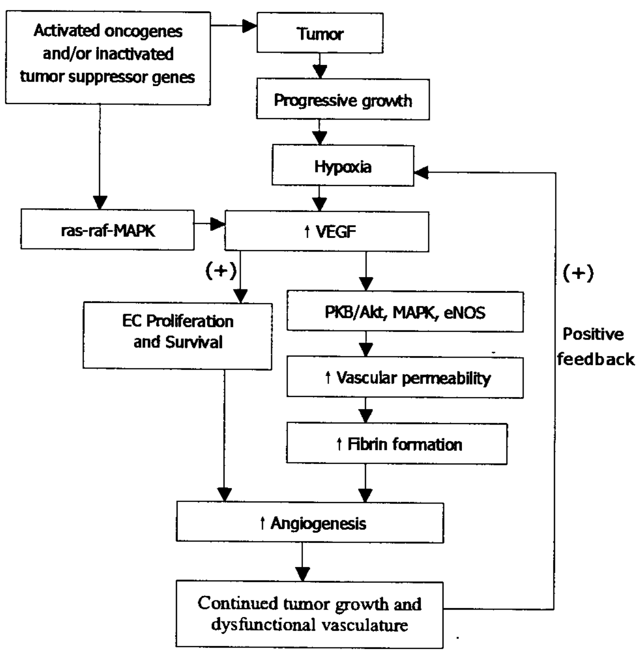


**Figure 1** The triggering mechanism in tumor angiogenesis: inactivated tumor suppressor genes/activated oncogenes versus hypoxia.

VEGF is an EC specific mitogen. VEGF, after binding to its high affinity receptors (Flt-1/VEGFR-1, Flk-1/KDR/VEGFR-2), promotes the formation of the second messenger via hydrolysis of inositol, thus induces the autophosphorylation of the receptors in the presence of heparin-like molecules, and open phosphatidylinositol metabolic signal transduction pathways, activates MAP kinases in EC and thereby VEGF exerts its mitogenic effect by promoting EC proliferation[8,9].

VEGF induces a balanced system of proteolysis that can remodel ECM components necessary for angiogenesis. VEGF stimulates EC production of urokinase-like plasminogen activator (uPA), tissue type plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1)[10,11], proteolytic enzymes, tissue factors, and interstitial collagenase[12]. Plasminogen activators activate plasminogen to plasmin, which can break down ECM components. In addition to remodeling the basement membrane, uPA bound to uPAR also mediates intracellular signal transduction in ECs. Tang *et al.* have demonstrated that uPAR occupancy on ECs results in the phosphorylation of focal adhesion proteins and the activation of MAP kinase[13] through which uPA influences EC migration and proliferation (Figure 2).

Moreover, VEGF has been shown to exhibit its angiogenic effect by inducing expression of the $\alpha_1\beta_1$, $\alpha_2\beta_1$ and $\alpha v\beta_3$ integrins, which promote cell migration, proliferation and matrix reorganization (Figure 2), and $\alpha_1\beta_1$, $\alpha_2\beta_1$ and $\alpha v\beta_3$ antagonists may prove effective on inhibiting VEGF-driven angiogenesis associated with cancers and other pathologies through apoptosis[14,15]. VEGF, in addition to a very specific mitogen for vascular EC, is a potent pro-survival factor for ECs in newly formed immature vessels. Several endothelial survival factors (VEGF, angiopoietin-1 and $\alpha v\beta_3$) suppress p53, p21, p16 and p27, and proapoptotic protein Bax, whereas they variably activate the survival PI3k/Akt, p42/44 MAP kinases, bcl-2, A1 and survivin pathways[16-20](Figure 2). It was reported that p42/p44 MAP kinases promoted VEGF expression by activating its transcription via recruitment of the AP-2/Sp1 (activator protein-2) complex on the proximal region (-88/-66) of the VEGF promoter and by direct phosphorylation of hypoxia-inducible factor 1 alpha (HIF-1 alpha)[21]. Pharmacological inhibition of PI3K or transfection with a dominant-negative Akt mutant abolished the antiapoptotic effect of VEGF on ECs. In addition to the PI3K/Akt pathway, ras-dependent signaling pathways might also play an important role at least for VEGF signaling. Thus, H-rasV12G down-regulation leads to profound tumor regression, which is initially characterized by massive apoptosis of tumor- and host-derived ECs[22]. Therefore, apoptosis induction is resistant to enforced VEGF expression, suggesting that VEGF requires an intact Ras-dependent signaling pathway to mediate its apoptosis inhibitory effect[22]. And also, VEGF via the KDR/Flt-1 receptor induces enhanced expression of the serine-threonine protein kinase Akt[19], a downstream target of PI3-kinase, which potently blocks apoptosis by interfering with various apoptosis signaling pathways[23,24], promotes EC migration[25], and enhances the expression of the hypoxia-inducible factor (HIF), which is known to stimulate VEGF expression[26], suggesting a potent proangiogenic effect[27,28]. These findings have identified the VEGFR2 and the PI3K/Akt signal transduction pathway as crucial elements in promoting EC survival induced by VEGF. The downstream effector pathways mediating the antiapoptotic VEGF effect include Akt-dependent activation of the endothelial nitric oxide synthase (NOS)[29,30], resulting in an enhanced endothelial NO synthesis, which, in turn promotes EC survival (Figure 2). Gupta *et al.* demonstrated that the VEGF-induced activation of the MAPK/extracellular signal-regulated kinase (ERK) pathway and inhibition of the stress-activated protein kinase/c-Jun amino-terminal kinase pathway is also implicated in the antiapoptotic effect mediated by VEGF[31] (Figure 2). Interestingly, the activation of the PI3K/Akt pathway mediates not only the antiapoptotic effect but also the migratory effect of VEGF on ECs via Akt-dependent phosphorylation and activation of eNOS[32] (Figure 2).

The expression of VEGF mRNA is highest in hypoxic tumor cells adjacent to necrotic areas. Hypoxia-induced transcription of VEGF mRNA is apparently mediated, at least in part, by the binding of hypoxia-inducible factor 1 (HIF-1) to an HIF-1 binding site located in the VEGF promoter, and by the activation of a stress inducible PI3K/Akt pathway[26,33]. In fact, progressive growth of tumor creates ongoing hypoxia, which up-regulates several pro-angiogenic compounds including VEGF, bFGF, IL-8, TNF-$\alpha$, TGF-$\beta$ etc. These compounds, via several mechanisms such as increase of vessel hyperpermeability, release of plasma proteins, induction of proteases, fibrin formation, EC proliferation, migration etc, promote angiogenesis and fibrinolysis resulting in continued tumor growth and dysfunctional vasculature, which further positively feedback to create continuing hypoxia inside tumors (Figure 1).

### Fibroblast growth factors

Fibroblast growth factors (FGFs) and their receptors are overexpressed in various types of cancers, and are important tumor angiogenic and ECs survival factors. Pardo *et al.* reported that bFGF induced expression of the antiapoptotic proteins bcl-XL and bcl-2 via the MEK/ERK signaling pathway[34] (Figure 2). Expression of VEGF mRNA in the tumor is increased by bFGF overexpression, and the bFGF-induced tumor development is significantly inhibited by treatment with KDR/Flk-1 neutralizing monoclonal antibody (mAb), which suggests that bFGF synergistically augments VEGF-mediated hepatocellular carcinoma development and angiogenesis, at least in part, by induction of VEGF through KDR/Flk-1[35]. In addition, bFGF induces an increase of VEGF mRNA in vascular smooth muscle cells[36] and an increase in VEGF receptors in microvascular ECs[37]. aFGF and bFGF are mitogenic for ECs and stimulate ECs migration as well as ECs production of plasminogen activator (PA) and collagenase that are capable of degrading basement membrane[38] (Figure 2). FGFs are responsible for production of ECM and release of matrix metalloproteinases (MMPs) for selective degradation and organization of ECM[39] (Figure 2).

Binding of FGFs to their high affinity receptors causes the activation of the intrinsic tyrosine kinase and a cascade of events, leading eventually to the induction of immediate early gene transcription, and to cell proliferation. FGFs receptors dimerize upon ligand binding, and transphosphorylate at tyrosine residue. Angiogenic growth factors, like bFGF and VEGF165, require interaction with heparin sulfate (HS) in order to induce a proliferative signal through tyrosine kinase receptors. Binding of bFGF to high affinity cell surface receptor sites can be modulated by heparin-mimicking compounds (i. e. RG-13577) that can modulate abnormal bFGF signaling by disrupting bFGF mediated autocrine loop, compete with heparin sulfate (HS) on binding to bFGF, bind the growth factor, and prevent receptor binding and/or dimerization[40], and by proteolytic enzymes (e.g. MMP-2) that cleave the ectodomain of the receptor. These effects are associated with profound inhibition of bFGF mediated signal transduction (tyrosine phosphorrylation) and proliferation of vascular ECs[40]. Spontaneous migration of ECs is inhibited by neutralizing antibodies to bFGF, suggesting an autocrine of bFGF synthesized and released by the ECs themselves[38]. A dominant-negative receptor, which, when co-expressed with

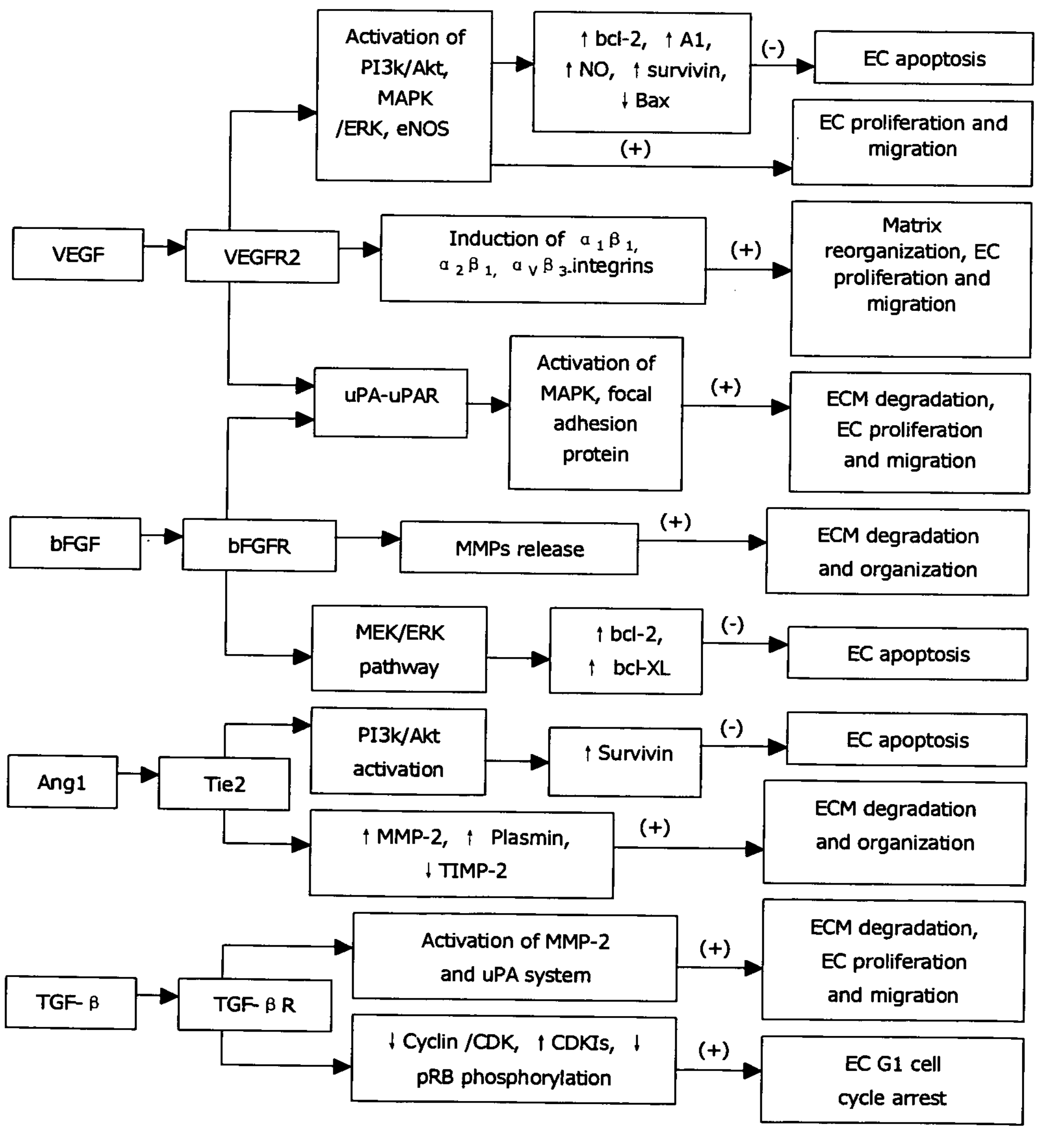


**Figure 2** Mechanism of angiogenesis by angiogenic factors.

FGF receptors (FGFRs), can block the activation and signal transduction. In addition, the ligand-specific targeting of toxin to tumor cells expressing FGFRs and the compounds that bind and inactivate FGF ligands, can block ECs proliferation.

### *Angiopoietins and tie receptors*

It has been proposed that angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2) are pro-angiogenic and anti-angiogenic owing to their respective agonist and antagonist signaling action through the Tie2 receptor[41]. Lobov *et al.* have demonstrated that *in vivo*, in the presence of endogenous VEGF-A, Ang2 promotes a rapid increase in capillary diameter, remodeling of the basal lamina, proliferation and migration of ECs, and stimulates sprouting of new blood vessels[41]. By contrast, Ang2 promotes ECs death and vessel regression if the activity of endogenous VEGF is inhibited[41]. It was reported that Ang1 induced phosphorylation of Tie2 and the p85 subunit of PI 3'-kinase and increased PI 3'-kinase activity in a dose-dependent manner, suggesting that the Tie2 receptor, PI 3'-kinase, and Akt are crucial elements in signal transduction pathway leading to EC survival induced by the paracrine activity of Ang1[42] (Figure 2). Alternatively, Ang1 prevents EC apoptosis via Akt/survivin pathway by activating a critical survival messenger, Akt, and by up-regulating a broad spectrum apoptosis inhibitor, survivin[43, 44] (Figure 2), but has no effect on the expression of bcl-2 and XIAP[44]. Moreover, Ang1-induced migratory effect might be mediated through PI 3'-kinase activity dependent tyrosine phosphorylation of $p125^{FAK}$, which plays a key role in regulating dynamic changes in actin cytoskeleton organization during EC migration[45]. Increased plasmin and MMP-2 secretion, and suppressed TIMP-2 secretion by Ang1 from ECs are also important determinants for inducing ECs sprouting[45] (Figure 2). In contrast, the PI 3'-kinase inhibitors have been found to inhibit Ang1-stimulated tyrosine phosphorylation of $p125^{FAK}$, and secretion of MMP-2 and plasmin from ECs and migration [45]. Ang2 blocks Ang1-mediated Tie2 autophosphorylation in ECs and acts as a check point on Ang1/Tie2-mediated angiogenesis to prevent excessive branching and sprouting of blood vessels by promoting destabilization of blood vessels.

### *Transforming growth factor-β*

Transforming growth factor-betas (TGF-βs) are multifunctional polypeptides that regulate cell growth and differentiation, ECM deposition, cellular adhesion properties, angiogenesis and immune functions. TGF-β1 acts through the TGF-β type I and type II receptors to activate intracellular mediators, such as Smad proteins, the p38 MAPK, and the ERK pathway[46]. TGF-β1 mRNA levels by activin receptor-like kinase 5 (ALK5) independent of p38 MAPK activation[46]. In contrast, TGF-β1 induction of fibronectin (FN) mRNA requires p38 MAPK activity[46]. TGF-β1 induction of PAI-1 and TSP-1 mRNA uses at least ALK5 and possibly the p38 MAPK pathway[46]. TGF-β secreted by most cultured cells is in biologically inactive form, and cannot bind TGF-β receptors; the latent TGF-β is activated by proteases such as plasmin and cathepsin D, low pH, chaotropic agents such as urea, and heat[47, 48]. Several studies suggested that VEGF increases plasminogen activator (PA) activity in vascular ECs[11] and that plasmin is able to activate latent TGF-$\beta_1$[49, 50] which decreases Flk-1 expression and thereby negatively regulates the VEGF/Flk-1 signal transduction pathway in EC[51], raise the possibility that a complex self-regulating mechanism of VEGF signal transduction may exist during angiogenesis[50]. However, immunohistochemical study has shown that TGF-β1 might be associated with tumor progression by indirectly stimulating angiogenesis through the up-regulation of VEGF expression in gastric carcinoma[52]. In addition, TGF-$\beta_1$ inhibits the generation of the anti-angiogenic molecule angiostatin by human pancreatic cancer cells in a time- and dose-dependent manner, and this effect is mediated through modulation of the plasminogen/plasmin system[53].

TGF-β not only inhibits the activity and expression of cyclins and CDKs but also induces the cyclin-dependent kinase inhibitors (CDKIs) p15, p27 and p15, which bind to the cyclin/CDKs, preventing phosphorylation of pRB and thereby arresting most epithelial cells (including ECs) at late G1 phase[54] (Figure 2). The effects of TGF-$\beta_1$ on endothelial tube formation may be mediated through a net antiproteolytic activity by modulating uPA and PAI levels[55]. Ellenrieder *et al.* reported that TGF-β treatment of PANC-1 and IMIM-PC1 cells resulted in strong up-regulation of expression and activity of both matrix metalloproteinase-2 (MMP-2) and the uPA system, and treatment with MMP inhibitors or inhibitors of the uPA system caused significant reduction of TGF-β-induced invasiveness in both cell lines suggesting that TGF-β acts in an autocrine manner to induce tumor cell invasion, which is mediated by MMP-2 and the uPA system[56] (Figure 2). Furthermore, TGF-β indirectly stimulates angiogenesis by the recruitment of inflammatory mediators that secrete angiogenic factors. Thus, TGF-β regulates vascular remodeling through its pleiotropic effects on different cell types.

### *Interleukin-8 and matrix metalloproteinase-2*

Up-regulation of MMP activity, favoring proteolytic degradation of the basement membrane and ECM, has been linked to tumor growth and metastasis, as well as tumor-associated angiogenesis. IL-8 mRNA is up-regulated in neoplastic tissues, such as non-small cell lung cancer[57] and that its expression correlates with the extent of neovascularization, tumor progression and survival. And also, MMP-2 mRNA level is increased in tumor cells transfected with IL-8, but VEGF and bFGF mRNA levels are unchanged[58,59] suggesting that IL-8-induced MMP-2 production is a major mechanism by which tumor cells induce angiogenesis. IL-8 can also be up-regulated by hypoxia, suggesting that the environment plays a major role in regulating IL-8 expression and metastasis[58]. MMPs induce tumor angiogenesis by degrading ECM and thereby release angiogenic mitogens that have been shown to be stored within the matrix. In addition, MMP-2 and MMP-3 are able to release soluble FGF receptor 1 (FGFR1)[60] and soluble 12-kDa immunoreactive and mitogenic heparin-binding epidermal growth factor (HB-EGF)[61], respectively. MMP-2 has been shown to directly modulate melanoma cell adhesion, spreading on ECM and invasion[62], and an inhibitor of MMP-2 significantly inhibits growth and neovascularization of tumors implanted into chick chorioallantoic membrane (CAM) by preventing MMP-2 binding to $\alpha v\beta_3$ and blocking cell surface collagenolytic activity[63]. Furthermore, MMP-9, as well as MMP-2 proteolytically cleave and activate latent TGF-β, and promote tumor invasion and angiogenesis[64].

### *Oncogene and tumor suppressor genes*

Oncogenes are found to be activated and tumor suppressor genes are found to be inactivated in tumor, and hence promote tumor growth and angiogenesis through different mechanisms (Figure 1). It has been shown that VEGF is introduced by K- or H-ras mutant gene, v-src and v-raf in transformed fibroblast and ECs. Other angiogenic factors such as VEGF, TNF-α, TGF-β have been shown to be up-regulated by mutant ras[65]. These effect may be mediated through a ras-raf-MAP kinase signal transduction pathway (Figure 1), which results in activation of promoter regions of genes of angiogenic growth factors[66]. Moreover, expression of ras, either constitutive or transient, potentiated the induction of VEGF by hypoxia[67].

p53 is an important suppressor gene, which inhibits the angiogenic process by inducing thrombospondin-1, down-regulating VEGF and NOS and, in addition, down-regulating hypoxia-induced angiogenesis, either inducing apoptosis or enhancing anti-angiogenic factors[68]. A transient transfection of mutated p53 results in up-regulation of VEGF mRNA in NIH3T3 cells[69]. In contrast, adenovirus-mediated wild-type p53 overexpression down-regulats CD40-induced VEGF expression and transmigration in human multiple myeloma cells expressing mutant p53[70]. And, we have previously demonstrated that the expression of Flt-1 receptor is significantly correlated with p53 mutation gene, not obviously with ras mutation gene in pancreatic carcinoma cells, which suggest that wild type p53, after mutation, might lose the suppressive function to the expression of Flt-1 receptor, thus results in neovascularization of pancreatic neoplasm and promotes the growth of tumor cells, whereas ras mutation may take part in neovascularization through other approaches. Recombinant wild type p53 represses bFGF mRNA translation in rabbit reticulocyte lysate, in a dose- dependent manner via blocking translation initiation by preventing 80S ribosome formation on an mRNA bearing the bFGF mRNA leader sequence[71]. Moreover, adenoviral vector-mediated wild type p53 transduction results in tumor regression, at least in part, via anti-angiogenesis mediated by the down-modulation of FGF binding protein, a secreted protein required for the activation of angiogenic factor bFGF[72]. In addition, wild type p53 gene transfer significantly reduces cell invasiveness *in vitro* via a decrease in the secreted levels of MMP-2 in mutated p53 human melanoma cell lines[73]. Biologically, p53 acts at a G1/S check point, postponding DNA replication after certain cell stress, such as DNA damage[74], and also induces the apoptotic pathway of cell death[75].

## THE ANGIOGENIC INHIBITORS

### *Mechanism of angiogenesis inhibitors*

Leading anti-angiogenic targets that have been identified are[76, 77]: (1) inhibition of the growth factors that promote endothelial proliferation; (2) inhibition of the proteases required for ECs to penetrate basement membrane and form new blood vessels; (3) disruption of specific intracellular signal transduction pathway; (4) induction of EC apoptosis or inhibition of EC survival; (5) inhibition of endothelial bone marrow precursor cells; and (6) inhibition of $\alpha v\beta_3$-integrin-vitronect interaction that is pivotal in mediating ECs adhesion to ECM during neovascularizatioin[77].

### *Inhibitors of angiogenic growth-factors and their receptors*

One broad class of angiogenesis inhibitors is made up of drugs that target growth factors such as bFGF and VEGF. The factors tend to bind to heparin, a property that may trap them within the ECM and may thereby govern their bioavailability. Hence, the early generation of drugs is heparin-like (e.g. Pentosan polysulfate), especially with regard to carrying multiple negative charges that promote growth factor binding. However, receptor targeting agents can impede tumor growth and metastasis by interfering, at specific growth-factor receptors, such as those for FGFs and VEGF, with the transduction of angiogenic stimuli into intracellular responses. In these pathways, the receptors are transmembrane tyrosine kinases, in which ligand binding to an extracellular domain induces autophosphorylation of an intracellular kinase domain. Each kinase then functions as an activator of downstream signals. To disrupt such a sequence, a drug may compete for receptor binding and prevent tyrosine kinase autophosphorylation. Inhibitors of VEGF family include: (1) anti-VEGF mAb[78]: directly neutralizes VEGF proteins, and inhibits biological activities of VEGF; (2) soluble VEGF receptors: specifically bind to VEGF, indirectly block the function of VEGF with receptors; (3) inhibitors of VEGF receptors[79]: bind to VEGF receptors and block their functions with VEGF; (4) inhibitors of VEGF signal transduction: interfere a series of signal transduction pathways by blocking autophosphorylation of VEGF receptors; (5) VEGF antisense[80]: is a specific nucleotide sequence, which binds to VEGF mRNA and thereby interferes VEGF mRNA translation and VEGF protein formation. A recent study has shown that the VEGFR2 DNAzyme can cleave its substrate efficiently in a concentration- and time-dependent manner, inhibit the proliferation of EC with a concomitant reduction of VEGFR2 mRNA, and inhibit tumor growth *in vivo*[81].

### *Endogenous angiogenesis inhibitors*

More than 40 endogenous angiogenesis inhibitors have been characterized, and they are divided into 4 major groups: interferons (IFNs), proteolytic fragments, interleukins (ILs), and tissue inhibitors of metalloproteinases (TIMPs)[82].

**Interferons** The interferons (INF-α, -β, and -γ) are members of a family of secreted glycoproteins, which have direct or indirect inhibitory effect on tumor angiogenesis and growth. IFN-α/β have been reported to down-regulate the expression of pro-angiogenic factor MMP-9 mRNA and protein in different cancers[83-86]. Also, IFN-α/β down-regulate IL-8 expression in bladder cancer[83-84]. Several studies demonstrated that the administration of optimal biological dose of IFN-α/β decreased the expression of bFGF mRNA and protein and microvessel density in the tumors and, in addition, induced EC apoptosis[83-85, 87]. Sasamura *et al.* demonstrated that IFN-γ had mild inhibitory effects on VEGF mRNA and bFGF mRNA expression, whereas IFN-α did not significantly decrease the level of either VEGF mRNA or bFGF mRNA in renal cell carcinoma[88]. However, some studies demonstrated that IFN-α/β treatment did not cause the reduction of bFGF and VEGF levels in serum from patients with carcinoid tumours[89] and leukemia[86]. Thus, anti-angiogenic effect of IFNs treatment might be mediated by the regulation of different angiogenic factors in different tumors in dose- and time-dependent manner. Moreover, IFN-γ is presumed to induce its anti-angiogenic effects through the secretion of IFN-γ inducible protein 10 (IP-10) and monokine induced by IFN-γ[90]. Finally, IFNs have antitumor properties, which may be mediated through a direct cytotoxic effect on tumor cells, augmentation of immunogenicity of tumor by up-regulation of major histocompatibility (MHC) classes *I and II* and tumor associated antigens, and/or activation of macrophages, T lymphocytes and natural killer cells[89].

**Interleukins** It was reported that interleukins (ILs) having a Glu-Leu-Arg (ELR) motif at the $NH_2$ terminus, such as IL-8, enhance angiogenesis, and those that lack this sequence, such as IL-4, inhibit it[91]. IL-4 inhibits *in vivo* neovascularization induced by bFGF in the rat cornea and blocks the migration of microvascular ECs toward bFGF *in vitro*[92]. However, it has been shown that IL-1α, a representative cytokine of activated macrophages, induces angiogenesis through the enhanced expression of various angiogenic factors such as VEGF, IL-8, and bFGF[93]. And also, IL-6 was found to counteract the apoptotic effect mediated by wild type p53[75]. Several studies have reported that IL-12 suppresses the expression of VEGF mRNA[94, 95], bFGF[94] and MMP-9 mRNA[94]. Additionally, IL-12 was found to stimulate mRNA expression of IFN-γ and its inducible anti-angiogenic chemokine IFNγ-inducible protein (IP-10) in ECs cultured with IL-12[95]. IL-12 significantly promotes apoptosis and inhibits proliferation rate of human tumors and extensive necrosis in the murine, and thereby reducing tumor vessel density[95]. Furthermore, the *in vivo* inhibition of neovascularization in IL-10-secreting tumors

might be mediated by the ability of IL-10 to down-regulate the synthesis of VEGF, IL-1β, TNF-α, IL-6, and MMP-9 in tumor-associated macrophages[96]. And also, IL-10 inhibits tumor metastasis through a natural killer (NK) cell-dependent mechanism[96].

**Tissue inhibitors of metalloproteinases** Remodeled ECM components comprise a scaffold upon which ECs can adhere, migrate, and form tubes, and deposition of these components forms the basal lamina that ensheaths endothelial and mural cells. *In vitro* migration of ECs through gelatin is significantly inhibited by overexpressed TIMP-1[97]. Murphy *et al.* reported that TIMP-2, but not TIMP-1, inhibited bFGF-induced EC proliferation[98]. TIMP-2 is able to inhibit soluble FGFR1 released by MMP-2[60]. Transfection of the highly metastatic B6F10 murine melanoma cell line with TIMP-2 cDNA showed the reduced levels of blood vessel formation and diminished induction of EC migration and invasion[99]. Studies have shown that the overexpression of TIMP-3 induces the apoptotic cell death of a number of cancer cell lines and rat vascular smooth muscle cells through the stabilization of TNF-alpha receptors on the cell surface, perhaps by inhibiting a receptor shedding metalloproteinase[100, 101]. Furthermore, anti-angiogenic and antitumor effects of TIMP-3 appear to be mediated, in part, by decreased expression of vascular endothelial (VE)-cadherin by ECs in the presence of TIMP-3 in an *in vitro* assay and in TIMP-3-overexpressing tumors[102]. Finally, TIMP-1, TIMP-2, TIMP-3 and TIMP-4 inhibit neovascularization by inhibiting MMP-1, MMP-2, and MMP-9 induced breakdown of surrounding matrix[103]. Thus, the multiple effects of TIMPs on both endothelial and tumor cells migration render MMPs attractive targets for tumor therapy.

**Proteolytic fragments** Most of these fragments are derived from ECM components, such as collagen or fibronectin, or from enzymes such as plasminogen and MMP-2 that remodel ECM. Perhaps the most characterized inhibitors in this class are angiostatin and endostatin.

**Angiostatin** The anti-angiogenic effect of angiostatin, a 38-kDa internal fragment of plasminogen, may be mediated, at least in part, by their ability to down-regulate VEGF expression within the tumor[104]. Angiostatin inhibits hepatocyte growth factor (HGF)-induced phosphorylation of c-met, Akt, and ERK1/2, and thereby exerts its anti-angiogenic effect via disruption of HGF/c-met signaling[105]. Intraperitoneal administration of angiostatin potently inhibits the neovascularization and metastasis formation in mice observed after a primary tumor has been removed[106]. It has been shown that binding of angiostatin to the α/β-subunits of plasma membrane-localized ATP synthase may suppress endothelial-surface ATP metabolism and thereby mediates its anti-angiogenic effects and the down-regulation of EC proliferation and migration[107, 108] (Figure 3). Further, adenoviral mediated angiostatin gene transfer selectively inhibits EC proliferation and disrupts the $G_2/M$ transition induced by M-phase-promoting factors, and that ECs show a significant mitosis arrest that is correlated with the down-regulation of the M-phase phosphoproteins[109]. Other studies have shown that angiostatin treatment significantly increases the apoptosis of EC and tumor cells, and decreases density of tumor blood vessels[109-111]. Angiostatin was found to produce a transient increase in ceramide that correlates with actin stress fiber reorganization, detachment and death[112] and, in addition, treatment with angiostatin or ceramide resulted in the activation of RhoA, an important effector of cytoskeletal structure[112] (Figure 3). Angiostatin can selectively regulate the expression of E-selectin and thereby inhibits the proliferation of ECs.

**Endostatin** It is a 20-kDa fragment of type XVIII collagen that has been identified as a factor produced by hemangioendothelioma cells that inhibits ECs proliferation, angiogenesis and tumor growth. The mechanisms by which endostatin inhibits VEGF-induced proliferation and migration of ECs are (Figure 3): First, endostatin blocks the VEGF-induced tyrosine phosphorylation of KDR/Flk-1 in ECs[113]. Second, endostatin suppresses the VEGF-induced activation of ERK, p38 MAPK, and p125[FAK], which are downstream events of the KDR/Flk-1 signaling and are involved in the mitogenic and motogenic activities of VEGF in ECs[113]. Third, endostatin inhibits the binding of VEGF to ECs and to its cell surface receptor, KDR/Flk-1[113]. Finally, endostatin directly binds to KDR/Flk-1 but not to VEGF[113]. Endostatin was found to exhibit its anti-migratory effect by reducing VEGF-induced phosphorylation of endothelial NOS (eNOS)[114] (Figure 3). Rehn *et al.* demonstrated that soluble endostatin was capable of binding to $\alpha_v$- and $\alpha_5$-integrins, thereby inhibiting the integrin functions, such as EC migration[115] (Figure 3). In addition, endostatin may exert its antiproliferative and anti-angiogenic effects by competing with bFGF for binding to cell surface heparan sulphate proteoglycans, which could disrupt the mitogenic growth factor signaling[116]. Endostatin induces a significant decrease in EC proliferation in the basal state and after stimulation by neuropeptide Y and bombesin[117]. Endostatin potently inhibits both the extracellular activation of proMMP-2 by inhibition of membrane-type 1 MMP (MT1-MMP) and the catalytic activity of MMP-2 and thereby can block the invasiveness of ECs and tumor cells[118]. The proapoptotic activity of endostatin appears to be mediated via tyrosine kinase signaling[119] and reduction of antiapoptotic proteins bcl-2 and bcl-XL without affecting the level of the proapoptotic Bax protein[120] (Figure 3). Furthermore, the Shb adaptor protein has been suggested to be involved in the mediation of the apoptotic signaling of endostatin[119] (Figure 3).

### *Somatostatin and its analogs*

Somatostatin (SS) and its analogs inhibit the proliferation of somatostatin receptors (SSTRs) positive endocrine neoplasm. The antiproliferative action of SS is signaled via five specific G-protein coupled receptors (SSTR1-SSTR5), which initiate pertussis toxin sensitive-G protein dependent, and tyrosine phosphatase mediated cell growth arrest or apoptosis according to receptor subtypes and target cells. It has been shown that activation of SSTR1, 2, 4, and 5 induce G1 cell cycle arrest through the ability of SS to maintain high levels of CDKIs $p27^{(Kip1)}$ and p21, and inactivate cyclin E-CDK2 complexes, thus leading to hypophosphorylation of pRb[121, 122](Figure 3). Moreover, somatostatin-mediated growth inhibition of normal and cancer pancreatic acinar cells is triggered via an inhibition of PI3-kinase signaling pathway[123]. SS may directly stimulate tumor apoptosis via sstr3-dependent G protein signaling, causing the induction of suppressor gene p53 and proapoptotic protein Bax[124](Figure 3). Our recent investigation reported that the low expression or loss of SSTR2 gene was more negatively correlated with the over-expression of p53 and ras mutation genes, which might take part in the angiogenesis of pancreatic neoplasm, whereas there was no significant relationship between SSTR2 and DPC4 (deleted in pancreatic cancer, locus 4), which suggested that there was different regulatory pathway in neovascularization of pancreatic neoplasm. Albini *et al.* provided evidence that SS inhibits Kaposi sarcoma associated angiogenesis by inhibiting both EC proliferation and invasion, and also by inhibiting migration of monocytes, which are important mediators of the angiogenic cascade, and are able to produce survival factors that, in turn, activate ECs[125]. In addition, SS induces a significant decrease in basal and stimulates EC proliferation in HUVEC, and also decreases number of capillaries[117]. CAM model study showed that unlabeled SS analogs inhibited angiogenesis, which was

proportional to the ability of the analogs to inhibit growth hormone (GH) production[126].

It is defined that SSTR subtypes are responsible for the specific post-receptor signal transduction mechanisms involved in octreotide' s inhibition of angiogenesis[126]. The intracellular signal transduction mechanisms involved in this angiogenic inhibition include the G^sub i^-binding protein, cAMP, and calcium[127]. Further, SS and its analogs induce their biologic effects by interacting with specific receptors that are coupled to a variety of signal transduction pathways involving adenylate cyclase, guanylate cyclase, ionic conductance channels, phospholipase C-β, phospholipase $A_2$, and tyrosine phosphatase and protein dephosphorylation and thereby regulate cell growth[128, 129]. The best characterized pathway involves the inhibition of adenylate cyclase, leading to a reduction in intracellular cAMP levels. Antiproliferative effects that are mediated through SSTR1 and SSTR2, involve the stimulation of tyrosin phosphatases, however SSTR5 appears to be coupled to inositol phospholipid/calcium pathway[130]. Mentlein *et al.* reported that cultivated cells from solid human gliomas of different stages and glioma cell lines secreted variable amounts of VEGF, which was reduced between 25 and 80 % of control levels depending on the glioma by co-incubation with SS or SSTR2-selective agonists (octreotide and L-054 522) in dose-dependent manner[131]. Growth factor-induced (EGF, bFGF) VEGF synthesis could also be suppressed to <50 % by co-incubation with SS or SSTR2-selective agonists, which was less pronounced in hypoxia-induced VEGF synthesis[131]. And also, SS and octreotide diminished the proliferative activity of cultured murine ECs HECa10 *vs.* controls; however, SS and octreotide did not change the release of VEGF into supernatants of 24-h or 72-h EC cultures[132]. A recent study has demonstrated that SS 14 can reduce bFGF-induced corneal angiogenesis[133].

In summary, the mechanisms of action of tumor growth inhibition by SS and its analogs are[134]: (1) inhibition of the secretion of hormones, such as GH, insulin and/or gastrointestinal hormones; (2) direct or indirect (via GH) inhibition of IGF-1 and/or other growth factors that exert a stimulatory effect on tumor growth. On the other hand, SS analogs can selectively stimulate the formation of IGF-binding protein 1, and thereby interfering with IGF-1 action at the receptor level; (3) inhibition of angiogenesis through different mechanisms; (4) direct antimitotic effects of growth factors, which act on tyrosine kinase receptors such as EGF and FGF, via SSTRs on the tumor cells; (5) modulation of immunological activity.

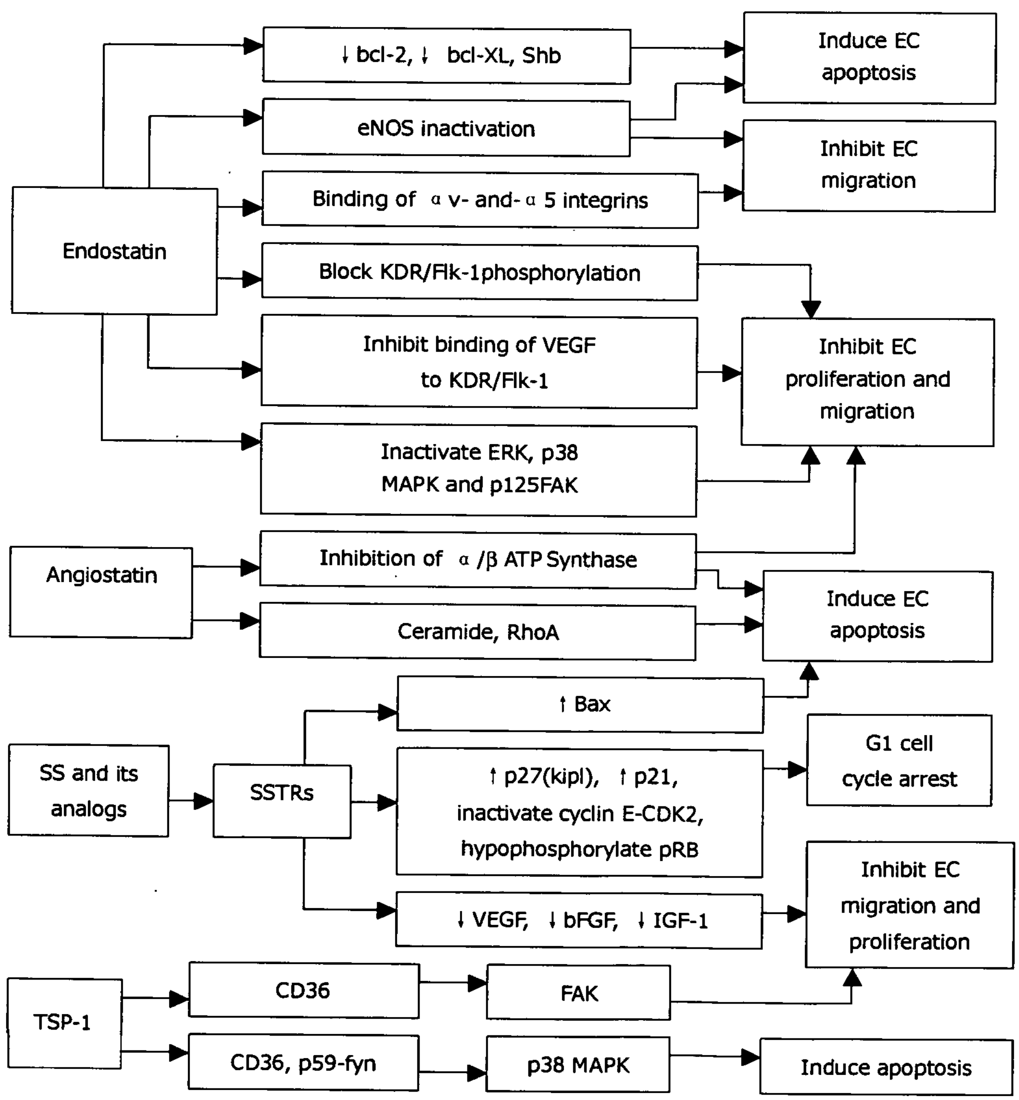


**Figure 3** Proposed effector pathways of angiogenic inhibitors.

### *Thrombospondin-1*

Thrombospondin-1 (TSP-1) is a naturally occurring inhibitor of angiogenesis that limits vessel density in normal tissues and curtails tumor growth. TSP-1 exerts its anti-angiogenic activity via binding to the CD36 receptor by triggering an apoptotic signaling pathway[135]. Binding of TSP-1 to CD36 receptor leads to the recruitment of the Src-related kinase, p59-fyn, and to activation of p38 MAPK. The activation of the p38 MAPK has been shown to be p59-fyn-dependent and to require a caspase-3-like proteolytic activity[135]. Furthermore, activated p38 MAPK leads to the activation of caspase-3 and to apoptosis[135] (Figure 3). Interestingly, the apoptotic effect of TSP-1 is restricted to ECs activated to take part in the angiogenic process and not in quiescent vessels[135]. TSP-1 acts through CD36 to modulate the activity of focal adhesion kinase (FAK) and thus inhibits EC migration and proliferation[136] (Figure 3). TSP-1 can effectively inhibit chemotaxis *in vitro* and neovascularization *in vivo*, induced by several angiogenic stimuli. These include protein that acts via tyrosine kinase receptors (VEGF, bFGF, aFGF, PDGF), via G proteins (IL-8), via serine/threonine kinase receptors (TGF-β), and also lipids (PGE-1)[137, 138].

## SUMMARY AND CONCLUSION

### *Developmental status and evaluation of anti-angiogenic therapy in human clinical trials*

Angiogenesis is a complex process that depends on the coordination of many different activities in several cell types. The angiogenic response in the microvasculature is associated with changes in cellular adhesive interactions between adjacent ECs, pericytes, fibroblasts, and immune mediators express many different cytokines and growth factors that react with other cells or ECM components to affect ECs migration, proliferation, tube formation, and vessel stabilization. As one or more of the positive regulators of angiogenesis are up-regulated, and simultaneously, certain negative regulators of angiogenesis are down-regulated, tumors become angiogenic. Interestingly, different angiogenic regulators, sometimes, function through the same mechanism and a single angiogenic regulator, sometimes, functions through different mechanisms. Hence, the anti-angiogenic therapy can be realized through the regulation of ' angiogenic switch' by interfering with different mechanisms.

Anti-angiogenic agents, if administered before a tumor develops or becomes vascular supply dependent, would therefore theoretically act similarly to a vaccine in preventing tumor development, not just tumor growth. However, it is notable that anti-angiogenic therapy represents a treatment, not a cure, for cancer. A cure for cancer can be realized only by targeting the agents and mechanisms that cause normal cells to become tumorigenic. The anti-angiogenic therapy of cancer, nonetheless, represents a highly effective strategy for destroying tumors because fundamental requirement of tumor growth is dependent on a blood supply. Unlike standard chemotherapy that targets tumor cells and other proliferating cells, angiogenesis inhibitors target dividing ECs that have been recruited into the tumor bed. For example, certain tubulin-binding agents such as combretastin A-4, exhibit a selective toxicity for proliferating ECs *in vitro* and causing a vascular collapse in tumor models *in vivo* via apoptosis and the subsequent death of much larger numbers of tumor cells[139]. Thus, specific anti-angiogenic therapy has little or no toxicities such as gastrointestinal symptoms and myelosuppression that are characteristic of standard chemotherapeutic regimens, does not require that the therapeutic agent enter any tumor cells nor cross the blood brain barrier, controls tumor growth independently of growth fraction or tumor cell heterogeneity or even tumor cell type, and does not induce acquired drug resistance [140]. Further, since normal vasculature in the adult is quiescent, the appropriate use of selective angiogenic inhibitors may be expected to confer a degree of specificity that is not obtainable with the nonspecific modalities of chemotherapy and radiation therapy and to allow for relatively nontoxic, long-term treatment of tumors.

Because anti-angiogenic agents are expected to be cytostatic rather than cytotoxic, they may be particularly effective in combination with cytotoxic agents, even used in advanced cases of pancreatic, colon, and hormone-refractory prostate cancer, thereby targeting not only DNA synthesis and cell division but also the biologic behavior of tumor cells. The following guidelines are suggested to improve the therapeutic efficacy of endogenous angiogenesis inhibitors in clinical trials: (1) after surgery or radiotherapy to prevent recurrence of distant metastases; (2) combinatorial therapies, for example, in combination with conventional chemotherapy, radiotherapy and vaccine therapy or immunotherapy, and also, in combination with several angiogenesis inhibitors rather than a single inhibitor; (3) targeting therapy. Angiogenesis inhibitors may be specifically targeted to the disease locus at high concentrations rather than be widely distributed in the entire body; (4) gene therapy, several advantages including prolonged therapy, low doses of DNA molecules, and less frequent injections may be achieved by anti-angiogenic gene therapy with endogenous angiogenesis inhibitors; (5) more potent angiogenesis inhibitors should be discovered; (6) prolonged half-lives. Slow-release of angiogenesis inhibitors in the body reaches a steady-state level in the circulation.

Remarkably diverse groups of anti-angiogenic drugs are currently undergoing evaluation in phase I, II or III clinical trials. However, there are still some difficulties associated with the clinical evaluation of these drugs efficacy. In the experimental animal model, tumors can be removed and examined for therapeutic efficacy such as changes in the extent of vascularization, vascular structure, EC viability or apoptosis, as well as for markers of angiogenic activity, e.g. VEGF expression. But in the clinical situation, taking serial biopsies of metastatic tumors may not be a particularly practical or desirable approach. For this, reliable surrogate markers of tumor angiogenesis in serum or urine, and non-invasive strategy may be necessary. Several studies have successfully used various non-invasive medical imaging strategies (e.g. MRI, Doppler ultrasound) to monitor changes in tumor blood flow, vascular structure and permeability[141-143]. Indeed, there are considerable research efforts underway in this field. In addition, there are obvious concerns about delayed toxicity associated with long-term anti-angiogenic therapy, and physiological angiogenesis affected by anti-angiogenic drugs such as wound healing in a cancer patient, reproductive angiogenesis (e.g. corpus luteum development in adult females, development of the vasculature in developing embryos), in neonates and children. In this concern, a potentially significant development in the near future could be the use of genomics based technologies to uncover a large number of highly (or even totally) specific molecular markers for the activated ECs of newly formed blood vessels.

In the near future, the outcome of ongoing clinical trials will give us more insights into the potential of anti-angiogenic approaches to treat cancer.

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# Clinical Targets for Anti-Metastasis Therapy

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Metastasis is responsible for most cancer deaths. Therapeutic strategies to prevent development of metastases thus have potential to impact on cancer mortality. Development of these therapies requires a better understanding of the biology and molecular events of the metastatic process. Metastasis is usually defined, clinically and experimentally, by evidence of the endpoint of the process, that is, the presence of metastatic tumors. Endpoint assays are suitable for determining if a therapeutic approach is effective, but can provide little information on how a treatment works *in vivo* and what steps in metastasis are affected. We describe here two methodological advances in the study of metastasis as a process: *in vivo* videomicroscopy, which permits direct observation of steps in metastasis, and a "cell accounting" technique that permits quantification of the fate of

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cells over time. These procedures have provided new and unexpected insights into the biology of the metastatic process. Based on these insights, we consider which steps in the metastatic process are biologically and clinically most appropriate as therapeutic targets for development of anti-metastasis therapies. We conclude that the most promising stage of the metastasis process for therapeutic targeting is the growth phase, after cancer cells have arrested in the microcirculation in secondary sites and have completed extravasation. Earlier phases in the process are either biologically inappropriate or clinically inaccessible, except in specific cases (e.g., chemoprevention strategies). The role of "seed" and "soil" in determining organ-specific metastasis is also discussed. The metastatic growth phase fortunately is a clinically broad target, and any treatment that limits growth of metastases prior to their causing irreversible harm to the patient has the potential to be clinically useful. A variety of therapeutic approaches to target this phase are under active development, including inhibition of angiogenesis or signal transduction pathways needed to support the growth of metastatic cells. 

## I. INTRODUCTION

In 1999, it is estimated that well over half a million Americans will die from cancer (Landis *et al.*, 1999). For the most part, these deaths will be due to the physiologic consequences of the growth of metastatic tumors, rather than to the effects of the primary tumor. Patients whose primary tumors have been detected before metastatic cells have been seeded to distant sites can generally be cured. However, these patients often cannot be readily identified. Two major difficulties in cancer treatment are, first, determining whether the tumor has indeed seeded metastatic cells at the time of detection, and second, how best to treat patients in which the metastatic process has (or is likely to have) already begun. In some cases, overt metastases are detected at the time of presentation. In others, however, no metastases can be detected, and the decision of whether metastasis has already been initiated in a given patient is based on probabilities, derived from a variety of prognostic indicators that have been shown, in other patients with similar tumors, to predict for future development of metastases.

There continues to be a great need to refine the predictive process for development of metastases, in order to more precisely assign patients to groups that likely require, or can be spared, further treatment following surgical removal or other treatment of the primary tumor. Furthermore, when a patient is identified by prognostic indicators as someone in whom metastasis is likely to occur, the treatment options to prevent metastases from developing are in many cases less than optimal. Similarly, the treatment options for patients in which overt metastases are present are often limited or less effective than one would like. For these reasons, metastases continue to be the primary source of cancer mortality. There thus remains a huge need for improved ways to treat existing metastases, in order to prevent their physiologic consequences to the patient, and to treat undetected metastases



believed to be present, to prevent them from growing and causing harm to the patient.

To achieve the goal of developing effective treatments to prevent mortality and morbidity due to metastasis, a better understanding of both the biology and the molecular biology of the metastatic process are needed. The steps in the process, and their timing over the course of the natural history of the disease, must be clarified. Similarly, molecules that contribute to the process of metastasis, and the steps at which each plays an important role, must be understood. This information is necessary for the development and appropriate clinical use of new anti-metastatic therapeutics.

## II. METASTASIS: CLINICAL AND EXPERIMENTAL CONSIDERATIONS

Metastasis is defined clinically by the "endpoint" of the process, the detection of secondary tumors at a site distant from the primary tumor. These tumors can be detected either on the basis of the effects that they cause to the patient (e.g., bone pain, in breast cancer that has spread to and grown in bone), or by detection by various medical imaging procedures (e.g., radiologic detection of metastases in internal organs), whether or not they are causing symptoms to the patient. The general steps in the process can be logically inferred, and include growth and vascularization of the primary tumor, escape of cells from the primary into the blood or lymphatic circulations (intravasation), transport of cells to distant organs, extravasation (escape from the circulation into the new tissue), and growth and vascularization of the new metastatic tumor. However, the details of these steps cannot be deduced from detection of this endpoint (i.e., presence or absence, and numbers, of metastatic tumors).

Experimentally, most commonly used assays are similarly designed to detect the endpoint of detectable tumors in distant organs (reviewed by Welch, 1997). In *spontaneous metastasis assays,* a primary tumor is produced by injecting cancer cells into an appropriate experimental animal (e.g., mice syngeneic to a murine cancer cell line, or immune deficient nude or SCID mice for various human cancer cell lines or immunogenic murine lines). Cells can be injected subcutaneously (i.e., an ectopic site for most tumor types) or, better, orthotopically (e.g., mammary fat pad injections for breast tumor cells) (reviewed by Fidler, 1991; Hoffman, 1994; Price, 1994; Killion *et al.*, 1999). Various transgenic tumor-prone mice also can be used, in which primary tumors spontaneously arise, and metastasis from these tumors can be assessed (reviewed by Christofori and Hanahan, 1994; Eccles *et al.*, 1994; Dankort and Muller, 1996). Metastatic tumors at sites distant from the implanted or spontaneously arising primary are then detected, at a single point in time, generally by dissection of the organs and observation and counting of metastases. Cells are assumed to have left the primary tumor and traveled to sec-

ondary organs, via the blood or lymphatic circulations, and to have grown to form detectable metastases in these sites. In contrast, the *experimental metastasis assay* is designed to model the latter half of this process by injecting cancer cells directly into the circulation of experimental animals and again detecting the endpoint of the presence and numbers of metastatic tumors in various internal organs such as lung or liver.

Both of these assays are very well suited for determining the effect of genetic, molecular, or therapeutic manipulations on the endpoint being quantified. Compounds or genetic manipulations of cells that give rise to decreased or increased numbers of metastatic tumors are clearly involved in the metastatic process, and this information is extremely valuable in defining factors that affect metastasis and may thus be targets for inhibition. Much progress has been made in recent years, using such endpoint assays, in identifying molecular and genetic factors that contribute to metastasis. However, metastasis assays that detect the endpoint of metastasis are by their very design poorly suited to clarifying the nature of metastasis as a *process*. The functional contributions of molecules identified as affecting the endpoint of metastasis cannot be directly assessed by such assays. Thus the roles these molecules play in the process have often been logically inferred rather than assessed experimentally.

For example, many studies had clearly implicated various classes of proteolytic enzymes in metastasis. A reasonable inference was that a major functional contribution of these enzymes to the process was by facilitating extravasation, the escape of cancer cells from the circulation in secondary organs, because the basement membrane lining the vessels was considered to be a significant physical barrier to the passage of cells. This assumption (based on logical deduction of what these enzymes must do *in vivo*) turned out to be a simplification of the much more complex role these enzymes play in the process, when this assumption was tested using procedures that allow observation of metastasis as an ongoing process (reviewed by Chambers and Matrisian, 1997). When murine melanoma cells were transfected to express the matrix metalloproteinase (MMP) inhibitor TIMP-1, both their metastatic ability *in vivo* and invasiveness *in vitro* were significantly reduced (Khokha *et al.*, 1992a,b; Khokha, 1994). Thus we expected to see a reduced ability of the cells to extravasate *in vivo*. However, the TIMP-expressing cells extravasated in the same proportions and with the same kinetics as did the parental cell line, when directly observed using *in vivo* videomicroscopy (Koop *et al.*, 1994, 1995). By direct observation of metastasis as a process, we were able to determine that this molecular intervention resulted in altered growth properties of the cells, after they had completed extravasation. A similar conclusion was reached when the anti-MMP agent batimistat was used to treat mice injected with melanoma cells: No alteration in extravasation was observed, whereas the metastases that formed were smaller, associated with a reduction in angiogenesis in the metastases (Wylie *et al.*, 1999).



Thus, the ability to observe metastasis as a process, rather than just as an endpoint, provides insights into how molecular and therapeutic interventions contribute to inhibition of metastasis. By relying on endpoint assays alone, one obtains a static view of metastasis. *In vivo* videomicroscopy, discussed in the next section, provides the opportunity to observe metastasis as a process.

A complete understanding of metastasis as a process also requires the ability to quantitatively follow the fate of cells during the process. Observations of cells at any point in the process need to be related to numbers of cells that began the process. To address this need, we have developed a new approach of "cell accounting," through which we can monitor and quantify the fate of a population of cells over time following their injection into an experimental animal, by providing a fixed reference point to the number of cells originally injected. Together, the procedures of *in vivo* videomicroscopy, coupled with a quantitative monitoring of the fate of cells over time afforded by the "cell accounting" approach, have offered new insights into the steps involved in metastasis, their relative ease or difficulty, and their relationship to the overall malignant phenotype of the cells. These methodological approaches, and results achieved using them, are discussed in the following sections.

## III. NEW TOOLS FOR STUDYING THE METASTATIC PROCESS

### A. *In Vivo* Videomicroscopy

Direct observations of experimental metastasis as it occurs over time may be carried out in a number of different organs and tissues by means of *in vivo* videomicroscopy (Fig. 1). Cancer cells can be fluorescently labeled *in vitro*, then injected into an animal and viewed at later times *in vivo* in thin tissues or superficial ($\leq 50\ \mu m$) regions of thick tissues by both fluorescence and oblique transillumination. Fluorescence provides for positive identification of cancer cells and speeds the initial process of finding them within a field of view. Oblique transillumination then permits individual cells to be viewed at high magnification in relation to their immediate environment. Because detailed descriptions of the methodology have been provided elsewhere (Chambers *et al.*, 1995; MacDonald *et al.*, 1998), only a brief summary is given here.

To locate cancer cells in tissues by *in vivo* videomicroscopy, high levels of cellular fluorescence are needed. Exogenous cytoplasmic markers such as Cal-

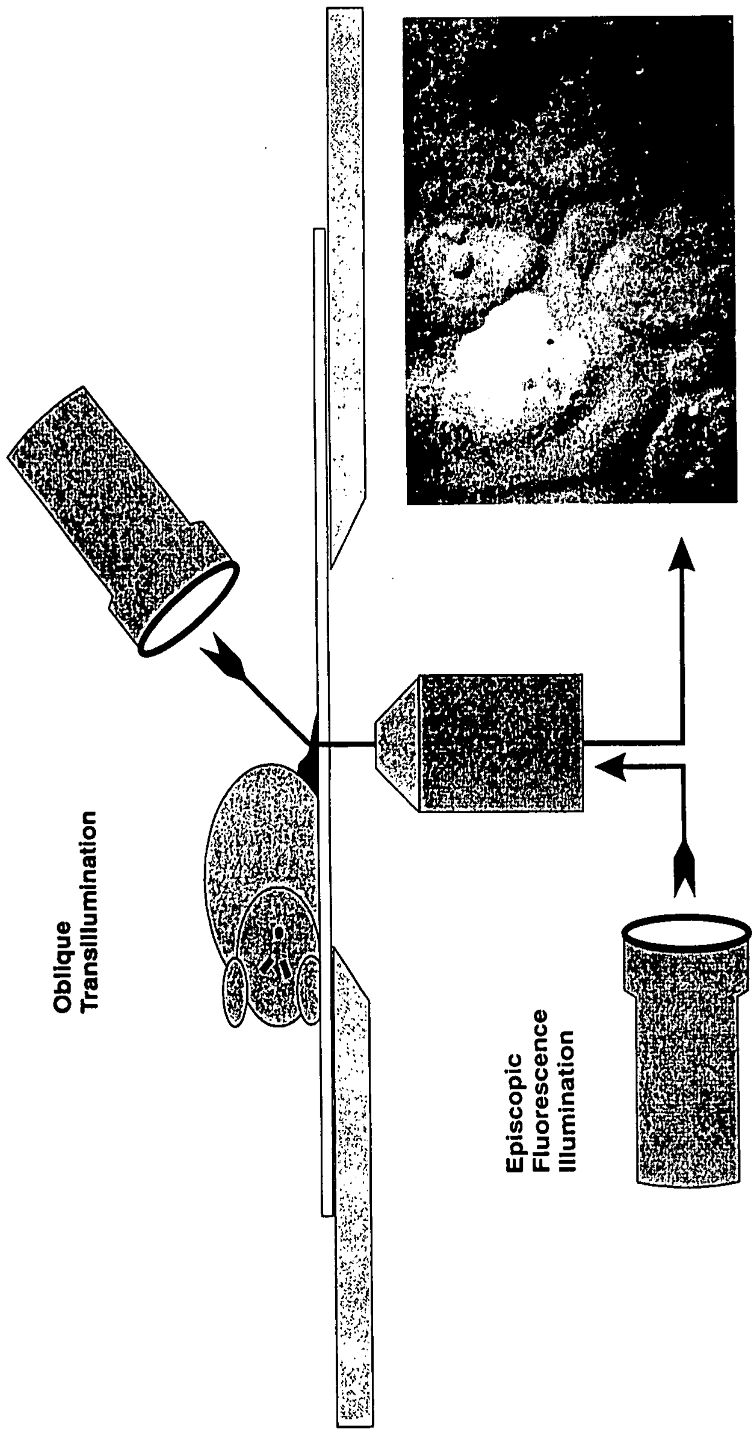


**Fig. 1** Schematic diagram of *in vivo* videomicroscopy method. The mouse is placed on the platform of an inverted microscope, the exposed intact organ (e.g., liver) being visualized through a glass coverslip using 10–100× objective lenses. Oblique transillumination via a fiber optic light guide and/or episcopic fluorescence illumination with appropriate filter blocks is used to observe fluorescently labeled cells within the organ. Images are viewed using a videocamera and monitor, and recorded on SVHS tapes and/or sent to a computer image capture system for further analysis.

cein-AM or fluorescent "nanospheres" 0.05–0.07 μm in diameter, which are internalized by the cells, are effective for this purpose (Morris *et al.*, 1994). Both markers leave cellular membrane integrity and growth potential unimpaired. [We found that use of nuclear fluorescent markers such as acridine orange is toxic to cells and results in membrane disruption and cell lysis (Morris *et al.*, 1993).] Calcein-AM gives a clear outline of the cell profile (MacDonald *et al.*, 1992), but is gradually lost from the cells and useful only for periods of up to 24 hr. Fluorescent nanospheres do not fade with time but become diluted at each successive cell division, such that after two or three divisions the marker can no longer be detected; however, undivided (i.e., dormant) cells can remain brightly fluorescent several weeks later. Recently, transfection of cells to express green fluorescent protein (GFP) has provided a heritable endogenous cytoplasmic marker that yields excellent views of cells and their pseudopodia (see Fig. 3 later), and makes long-term studies of metastatic growth possible (Chishima *et al.*, 1997; Naumov *et al.*, 1999).

After labeling, cells are injected intravascularly so as to target the organ being studied, for example, mesenteric vein for liver, chorioallantoic membrane (CAM) vein for chick embryo CAM. [Other organs such as lung (Cameron *et al.*, 2000), gut, spleen, and pancreas may also be studied, as described by Chambers *et al.* (1995).] At selected times later, a superficial layer of the partially exteriorized organ with its blood flow intact is examined microscopically *in vivo* (Fig. 1). For this purpose, the animal is placed on the stage of an inverted microscope with the organ resting on a glass coverslip above the objective lenses (10–100×, dry). In this way, the lower surface of the organ remains stationary and within the plane of focus, in spite of any respiratory motion to which the rest of the tissue is subjected. Epifluorescence illumination is used to locate cancer cells, followed by or in conjunction with transillumination to obtain high-resolution images and see cells clearly in relation to their immediate surroundings.

Transillumination is best carried out using obliquely incident light via a fiber optic guide. This arrangement results in more light being refracted from one side of the cells than the other and produces a shadowing effect, greatly enhancing the contrast and imparting a three-dimensional quality to the image. Cells are then seen by virtue of their shape rather than their optical density. In an organ such as the liver, the large volume of blood in the path of the incident light gives rise to a monochromatic red image that is extremely difficult to view. However, a Newvicon tube video camera with extended red sensitivity (Panasonic WV1550; Hamamatsu C2400) attached to the microscope yields high-contrast, nongrainy black-and-white images that can be viewed comfortably on the monitor and recorded on SVHS tapes, or captured by computer, for later analysis. By focusing up and down, "optical slicing" of the tissue may be carried out, due to the shallow depth of focus at high magnification (~1 μm for a 100× objective).

A limitation of *in vivo* videomicroscopy is that observations are restricted to a superficial layer of tissue 50–75 μm in thickness. For chick CAM this presents no problem since the total thickness of the tissue is less than this value. In mouse liver, studies using thick sections through the whole organ show that there is good reason to focus on this superficial layer of tissue, for it is at the surface of the organ, specifically, that metastases from various cell lines develop (Luzzi *et al.*, 1998; Naumov *et al.*, 1999). By *in vivo* microscopy one can study the metastatic process as it occurs over time, as well as the effects of molecular interventions on specific steps in metastasis. Using the same technique it is also possible to study the events of lymphogenous metastasis, as we have recently begun to do (Trites *et al.*, 2000).

## B. Cancer "Cell Accounting" in Tissues

We have devised a new experimental procedure to quantify accurately the survival of injected cancer cells in tissue, at successive early steps in hematogenous metastasis (Koop *et al.*, 1994; Luzzi *et al.*, 1998). To determine survival at any particular instant, the number of cancer cells actually observed in a sampled volume of tissue must be expressed relative to the number of cells that originally entered that volume. Our approach, based on the standard method for measuring distribution of blood flow (Rudolph and Heymann, 1967), is to include in the cell suspension injected, inert plastic ~10-μm microspheres at a known ratio, for example, 1 microsphere per 10 cells. This cell-to-microsphere ratio was chosen to minimize the effects of capillary blockage. After injection, the microspheres become trapped by size restriction in blood capillaries (or liver sinusoids) and remain in the tissue indefinitely, since they do not extravasate or deform to pass through the microcirculation. Thus, the total number of microspheres present in any particular region of tissue provides a reference marker for the total number of cancer cells which originally entered that region. On this basis it may be seen that the percentage of cells surviving in the tissue at a particular time after injection (Fig. 2) is given by the ratio of cells to microspheres present in the tissue, divided by the corresponding ratio present in the syringe immediately prior to injection, ×100%.

This method may be used in conjunction with *in vivo* videomicroscopy to

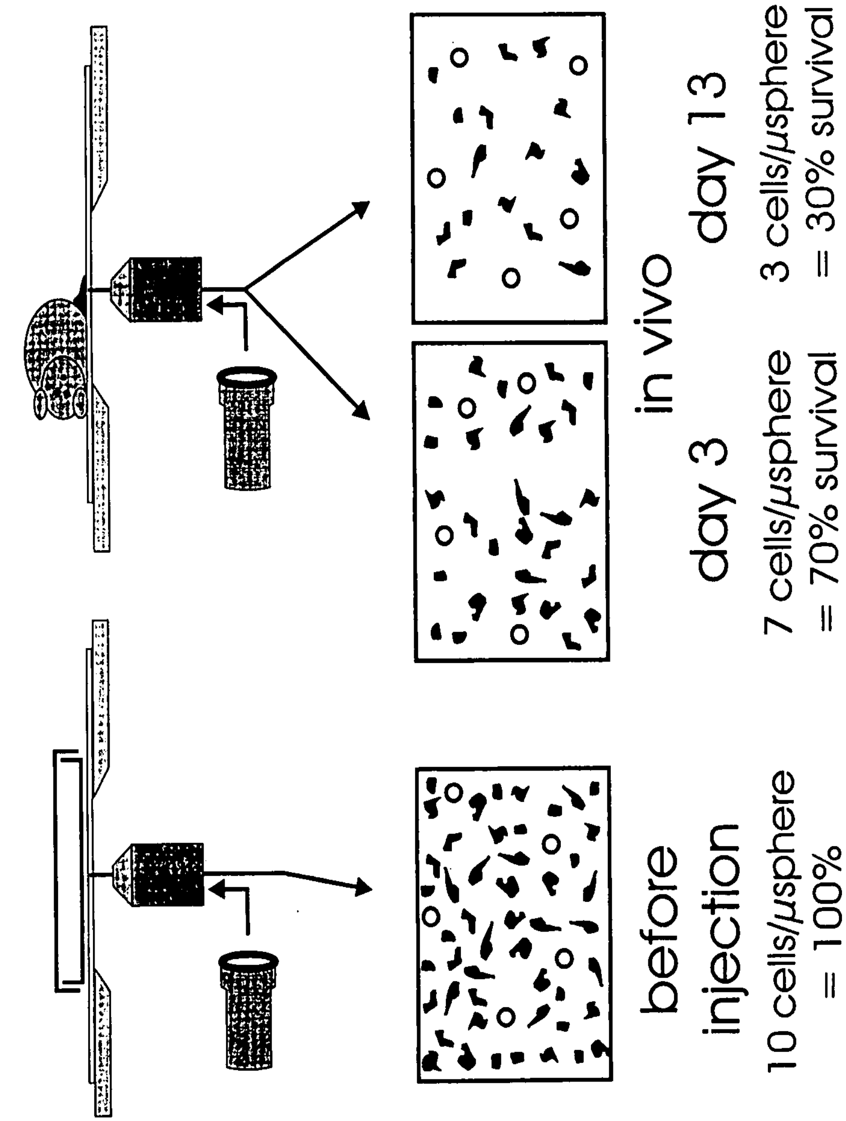


**Fig. 2** "Cell accounting" procedure for quantifying survival *in vivo*. The cell-to-microsphere ratio in the suspension is determined before injection and in the tissue of the target organ at various times after injection. The microspheres remain trapped in the tissue indefinitely, providing a reference marker for the number of cells delivered to that tissue. From the cell-to-microsphere ratios at later times, the percentages of surviving cells can be determined (see example provided).

sample superficial regions of the tissue, or after fixation the tissue may be sampled throughout its entire thickness by counting cells and microspheres in 30- to 50-μm-thick sections of tissue (Luzzi *et al.*, 1998). Because metastases have been shown to be clonal in origin (Talmadge *et al.*, 1982; Chambers and Wilson, 1988), the same method may be used to quantify them as well. When used together, *in vivo* videomicroscopy and our cell accounting procedure provide, for the first time, a means to determine at sequential times after injection the proportions of injected cancer cells that extravasate and survive in the tissue, remain as solitary dormant cells, form micrometastases, or develop into tumors. Such information, in turn, opens the way to dissect out the separate contributions to metastatic inefficiency from each of the sequential steps in the metastatic process (Luzzi *et al.*, 1998).

### C. Significance of the Procedures Described

What has been lacking in experimental metastasis methodology until now is the means to directly watch steps in the metastatic process as they occur *in vivo* over time, and to quantify the percentages of injected cancer cells surviving after each of these steps. The *in vivo* videomicroscopy and cell accounting procedures described in the preceding sections, coupled with fluorescence labeling for unambiguous identification of cancer cells *in vivo* or in thick sections of fixed tissue, provide new tools for studying early steps in the metastatic process. The use of these experimental approaches in combination with standard histologic and immunohistochemical procedures has led to a new conceptual understanding of early steps in metastasis and the contributions of individual steps to metastatic inefficiency. These approaches can now be used to determine which steps in metastasis are affected by a particular treatment or genetic manipulation, to test molecular mechanisms of metastasis, and to identify potential therapeutic targets.

## IV. NEW INSIGHTS INTO THE METASTATIC PROCESS

### A. Arrest and Survival of Cancer Cells in the Microcirculation

Using *in vivo* videomicroscopy, it is now possible to watch directly the arrival of blood-borne cancer cells in an organ and determine the way in which they become arrested within the microvasculature (Chambers *et al.*, 1992; MacDonald *et al.*, 1992; Morris *et al.*, 1993). It has become clear that in the organs and tissues we have studied, the arrest of cells from solid tumors oc-

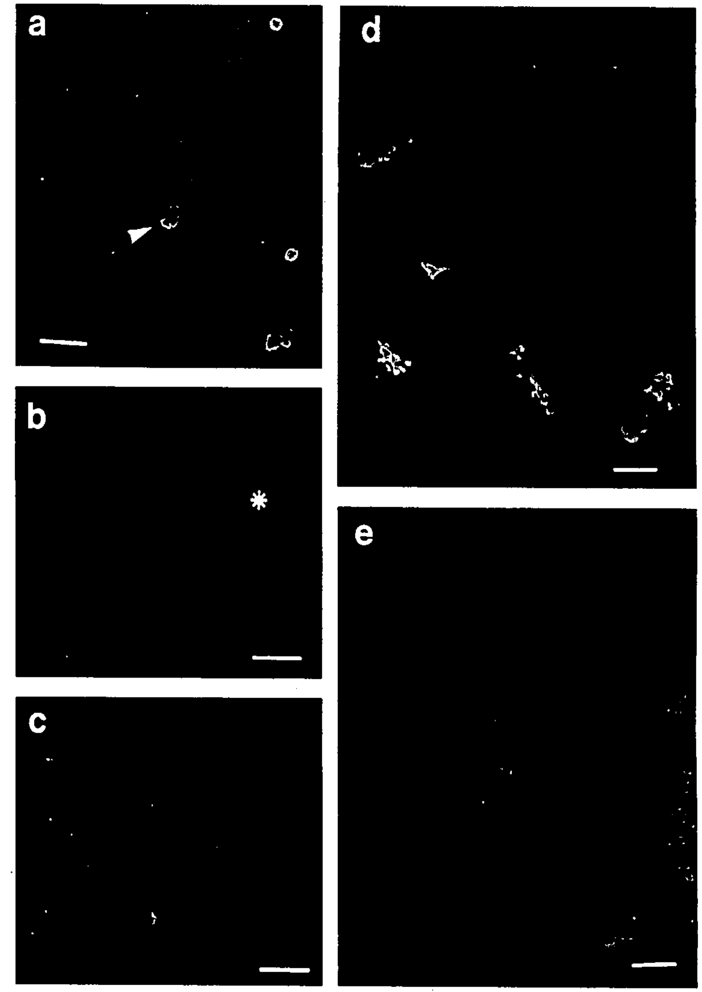


**Figure 3** Sequential steps of metastasis of GFP-expressing CHO cells in mouse liver, visalized using *in vivo* microscopy, using both transillumination and epifluorescence except where noted. (a) Intravascular cells arrested due to size restriction in liver sinusoids near terminal portal venules (acinar zone 1), seen 1 hr after injection via a mesenteric vein. Tumor cells appear bright green against the background of dark red blood vessels and pale yellow autofluorescence of liver tissue. Arrowhead indicates a cell deformed due to hemodynamic pressure. Bar=50 μm. (b) An extravasated cell 1 day after injection, in the process of migration to the hepatocyte layer in the subcapsular region of the liver. Cell body (*), slightly below the plane of focus, extends a long, bifurcated pseudopodial projection up to the liver surface. Bar=25μm. (c) Micrometastasis 4 days after injection, viewed by epifluorescence alone. Bar=25 μm. (d) Overview showing small micrometastases 4d after injection. Note variety of shapes and sizes, as well as pseudopodial projections at the periphery of most micrometastases. Bar=100 μm. (e) Larger micrometastases at 9 days after injection, with pseudopodial projections extending from their peripheries into the surrounding normal liver tissue. Bar=100 μm. (Republished from Naumov *et al.*, 1999.)

curs by quite different means from those by which leukocytes are arrested. Thus, leukocytes pass through blood capillaries or liver sinusoids and are arrested by adhesion to walls of venules much larger than the cell diameter, rolling along the endothelium before forming shear-resistant bonds. In contrast to this, cancer cells from solid tumors are much larger than leukocytes and are less able to pass through vessels of capillary dimensions to reach the venules.

Our direct observations show that cancer cells become arrested by size restriction in small vessels at the input side of the microcirculation in mouse liver and muscle (Fig. 3a, see color insert) and in chick CAM (Morris *et al.*, 1993, 1994; Koop *et al.*, 1994, 1996). Immediately after arrest, the cells undergo deformation due to hemodynamic pressures and conform to the boundary restrictions imposed by the vessel (Fig. 3a, arrow). The degree of deformation is modest in relatively low-pressure circulations such as CAM and the portal circulation of the liver, but under the higher hemodynamic pressures found in muscle the cells reach length-to-width ratios of up to 8:1. However, in spite of such severe deformations the cells do not become lysed but retain their membrane integrity, as shown by exclusion of ethidium bromide (Morris *et al.*, 1993). In CAM and liver we have studied these cells over extended periods of time and found that the vast majority of cells successfully extravasate into surrounding tissue within 1–2 days, which further attests to the continued viability of cancer cells in spite of hemodynamic forces and arrest in the microcirculation (Chambers *et al.*, 1995; MacDonald *et al.*, 1998).

Based on extrapolation from *in vitro* studies, it has been believed that arrest of blood-borne cancer cells in an organ or tissue is determined solely by specific adhesive interactions between cells and the endothelium. This concept has appeared attractive because it would provide a rational basis for organ specificity of metastasis. However, our observations from *in vivo* videomicroscopy have demonstrated that for the systems we have studied this view is no longer tenable. These findings lead us to propose that cancer cells which are blood-borne, after release from solid tumors, will become arrested initially on the basis of size restriction in the microcirculation of whatever organ or tissue they enter. This view accords with findings in mesentery by Thorlacius *et al.* (1997). It is interesting that whereas Scherbarth and Orr (1997) confirmed our findings in normal mouse liver, they also discovered that in mice pretreated with interleukin 1α (IL-1α) the cancer cells could become arrested by adhesion to walls of presinusoidal vessels twice the cell diameter. This suggests the possibility that release of cytokines into the bloodstream could bring about arrest of some cancer cells in portal venules, any cells that escape such arrest becoming trapped by size restriction in periportal sinusoids.

Metastatic inefficiency has generally been considered the result of a massive destruction of cancer cells within the circulation, due to the immune system and/or hemodynamic forces. Studies with $^{125}$IUdR-labeled cells in-

jected intravenously to target the lung showed a >100-fold reduction of radioactivity in the organ after 24 hr, suggesting that >99% of cells may have been destroyed (Fidler, 1970). Based on *in vitro* studies it was later proposed that such loss could arise if cells were arrested and deformed in blood capillaries, followed by stretching of their plasma membranes to the point of rupture (Weiss, 1987). Our own videomicroscopic studies, based on direct observation of cancer cells *in vivo,* following labeling *in vitro* with nontoxic fluorescent markers and injection into the circulation, demonstrate that the cells are indeed arrested and undergo deformation within capillaries or liver sinusoids (Fig. 3a). However, the vast majority of cells survive arrest and deformation, and go on to extravasate (Morris *et al.,* 1994; Koop *et al.,* 1994, 1995, 1996; Luzzi *et al.,* 1998; Wylie *et al.,* 1999). Similar results have been obtained for several different types of cells, including melanoma, mammary carcinoma, rhabdomyosarcoma, and normal and oncogene-transformed fibroblasts (Morris *et al.,* 1993, 1994, 1995; Hangan *et al.,* 1996; Luzzi *et al.,* 1998).

If these results from *in vivo* videomicroscopy can be translated to other vascular beds and cell types, as well as to the clinical situation, our findings suggest the new concept that most cells which escape from a solid tumor into the bloodstream (and go wherever the blood flow takes them) may not undergo rapid destruction within the circulation, but survive.

## B. Extravasation of Cancer Cells into the Tissues

Divergent views are to be found in the literature regarding the way in which cancer cell extravasation occurs. These views are derived from histologic and ultrastructural examination of tissues after cancer cell injection into the circulation, rather than from direct microscopic observations *in vivo.* The first view is that arrested cells replicate within vessels and, after proteolytic destruction of adjacent vascular basement membranes, extravasate *en masse* (Chew *et al.,* 1976; Crissman *et al.,* 1985, 1988). In contrast, another view is that cancer cells extravasate on an individual cell basis, similar to leukocytes, and with minimal disruption of the vessel wall. This is followed by replication within the tissue (Dingeman and Roos, 1982). It has been generally believed that the process of extravasation constitutes a barrier that few cancer cells successfully overcome.

We have used *in vivo* videomicroscopy in chick CAM and mouse liver to observe directly cancer cells in the process of extravasation (Chambers *et al.,* 1992, 1995; MacDonald *et al.,* 1992, 1998; Koop *et al.,* 1994, 1995; Morris *et al.,* 1994, 1995, 1997). No evidence for intravascular replication has been found and, invariably, cells extravasated singly without observable disruption of the microvasculature. For several hours after initial arrest in liver sinusoids, cancer cells completely obstruct the blood flow through the vessel segments concerned (Fig. 3a). Thereafter, cells gradually move away from one side of each vessel and extend themselves along the opposite wall, making possible a progressive resumption of blood flow (Morris *et al.,* 1994, 1995). This stage of the extravasation process is obviously dependent on the formation of shear-resistant bonds between cell and vessel wall, indicating that adhesion molecules (identity unknown) are playing a critical role. Each cell then forms pseudopodial projections, which extend out through the vessel wall and between the surrounding hepatocytes (Morris *et al.,* 1994). Finally, migration of the cell body occurs into the extravascular tissue, displacing hepatocytes and often wrapping around the abluminal surface of the sinusoid (Fig. 4a). In chick CAM the extravasated cells wrap around arterioles in the mesenchyme (Fig. 4c).



The first extravasated cells may be seen, in both CAM and liver, at between 2 and 3 hr after initial cell arrest in the microcirculation. However, for an entire population of cells to complete the process of extravasation takes considerably longer, since not all cells begin to extravasate at the same time. For each animal there is roughly a 3- to 5-hr time period during which observations by *in vivo* videomicroscopy can be carried out, and this permits the locations of large numbers of cancer cells to be assessed. Cells are classified as intra- or extravascular, or in the process of extravasation, and the percentages in each category are then determined. By studying a series of animals at successive times postinjection and combining the data, the time course of extravasation for an entire population of cancer cells can be obtained (Koop *et al.,* 1994, 1995). By 2–4 days after initial arrest in the liver microcirculation, virtually all of the injected cells are found to have extravasated (Morris *et al.,* 1994; Luzzi *et al.,* 1998; Wylie *et al.,* 1999).

The process of extravasation has been widely regarded as an important rate-limiting step in metastasis. This belief stems from (1) the recognition that degradation of vascular basement membrane and extracellular matrix is necessary for cancer cells to extravasate, taken together with (2) experimental results obtained from cell invasion assays through Matrigel *in vitro.* Based on differences in the extent of physical barriers in CAM capillaries (continuous endothelial lining and complete basement membrane by day 11, when cells were injected) versus liver sinusoids (fenestrated endothelium and incomplete basement membrane), one would predict that cells would extravasate with a shorter time course in liver than in CAM. However, the reverse is true, because it took ~18 hr in liver versus 7 hr in CAM for 40% of injected B16F1 cells to extravasate (Chambers *et al.,* 1995; MacDonald *et al.,* 1998). Clearly, the *in vivo* situation is more complex than that predicted from *in vitro* assays, and other factors such as differences in signaling molecules present in CAM versus liver must also play a major role in cancer cell extravasation.

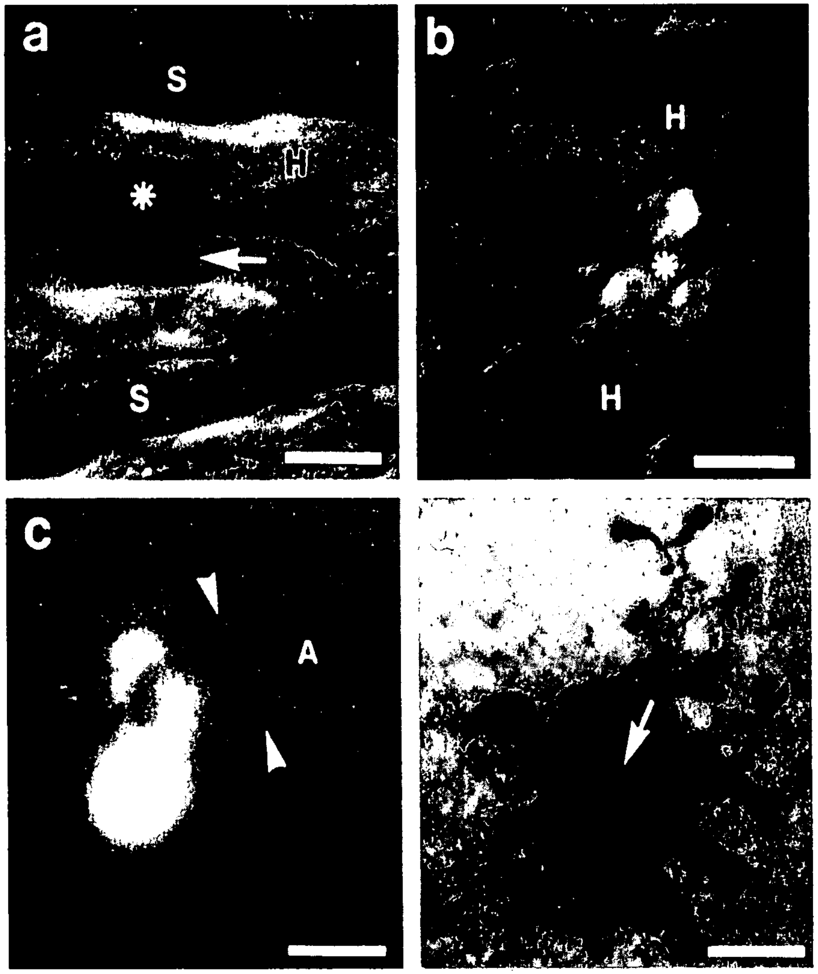


**Fig. 4** Views of cancer cells *in vivo* by videomicroscopy. All marker bars = 20 μm. (a) Melanoma cell (*) in mouse liver has completely extravasated and displaces hepatocytes (H), at 24 hr after injection. At a deeper plane of focus the cell wraps around a sinusoid (arrow, blurred in this image) in which rapid flow has resumed. S, sinusoids. (b) Melanoma cell (*) that has extravasated and migrated to the hepatocyte layer in the subcapsular region of mouse liver, by day 2. Note outlines of hepatocytes (H). (The fluorescent nanosphere labeling gives the cell a spotty appearance, and several pseudopodia are visible.) (c) Extravasated melanoma cell wrapping projections around an arteriole (A) in chick embryo chorioallantoic membrane, ~4 hr after injection. Arrowheads mark the arteriolar wall. (Photograph courtesy of Sahadia Koop.) (d) Micrometastasis, highly melanotic, has formed by 3 days after injection in chick chorioallantoic membrane. It is growing around a terminal arteriole (arrow).

Based on the view that extravasation is a major rate-limiting step in metastasis, it has been inferred that highly metastatic cells will extravasate more readily than cells of lower metastatic potential. However, our findings from



*in vivo* videomicroscopy do not support this view. We compared the time course of extravasation in mouse liver for two mammary carcinoma cell lines, from separate tumors both derived from D2 hyperplastic alveolar nodules. The first cell line (D2A1) is highly invasive in Matrigel invasion assays *in vitro* and highly metastatic in both chick CAM and mouse liver, whereas the second (D2.OR) is almost entirely noninvasive *in vitro* and poorly metastatic. To our surprise, we found that both cell lines extravasated with the same time course (Morris *et al.*, 1994). When similar experiments were carried out in chick CAM (Koop *et al.*, 1996), comparing the extravasation of (1) highly metastatic *ras*-transformed NIH 3T3 cells, (2) control nontumorigenic nontransformed NIH 3T3 cells, and (3) primary mouse embryo fibroblasts, we found that all three cell lines extravasated with the same kinetics (Fig. 5). By 24 hr after injection and arrest in the microcirculation, more than 89% of observed cells had completed the process of extravasation.

Thus, a new key concept from direct observations of cancer cell extravasation *in vivo* is that highly and poorly metastatic cells, or even nonmetastatic cells, may extravasate equally well. Our evidence from both chick CAM and mouse liver supports the view that most blood-borne cancer cells, whether metastatic or not, can successfully extravasate into surrounding tissue after arrest in the microcirculation. If these results can be translated to other vascular beds and cell types, our findings suggest that, contrary to previous belief, the process of extravasation does not represent a major barrier for cancer cells. Therefore, extravasation can no longer be considered a rate-limiting step in metastasis.

Proteinases and integrins are known to play important roles in the metastatic process, based on endpoint analyses such as number and size of metastases. However, which particular steps in metastasis are affected most by these molecules has remained unknown. Using *in vivo* videomicroscopy together with our recently developed cell accounting assay, we have found (Koop *et al.*, 1994, 1995) that overexpression of metalloproteinase inhibitor in B16F10 cells does not affect extravasation but reduces tumor growth in chick CAM. At 24 hr after injection, more than 80% of injected cells of both cell lines survived and had successfully extravasated. Similar results have been obtained for B16F1 cells in mouse liver, using the synthetic metalloproteinase inhibitor batimastat (Wylie *et al.*, 1999). These studies indicated that MMP inhibitors, both endogenous and exogenous, produced a reduction in growth of metastases that was caused by effects on postextravasation events. In the case of batimastat, this reduction in growth occurred in conjunction with an inhibition of tumor angiogenesis (Wylie *et al.*, 1999). Other *in vivo* microscopy experiments that focused on the role of integrins in metastasis, also showed tumor inhibition because of effects on postextravasation cell migration and growth, with little or no effect on the process of extravasation itself (Morris *et al.*, 1995; Hangan *et al.*, 1996).

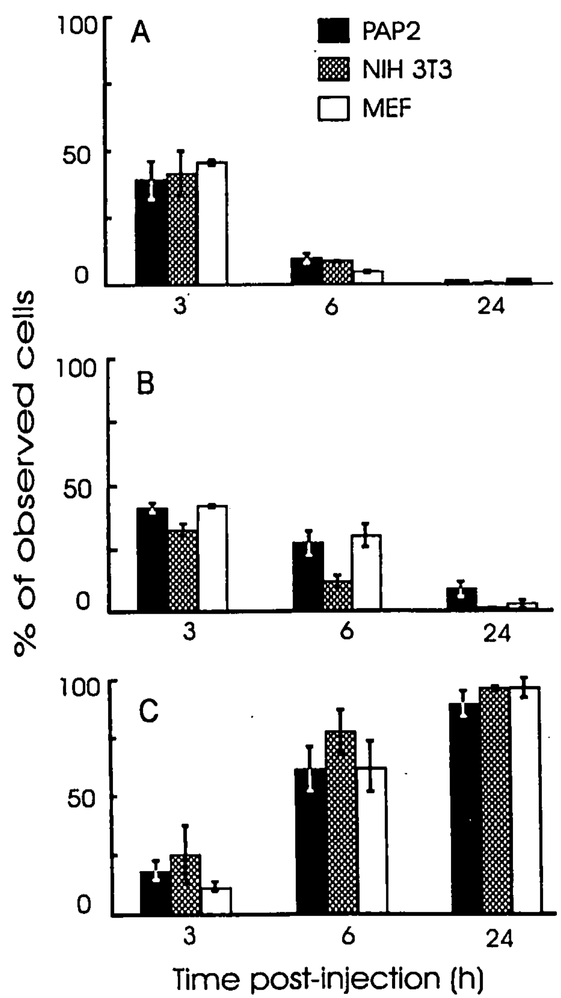


**Fig. 5** Kinetics of extravasation of highly metastatic, *ras*-transformed NIH 3T3 cells (PAP2), control nontransformed NIH 3T3 cells, and primary mouse embryo fibroblasts (MEF) from the chick embryo CAM microcirculation. Percentages of cells that were intravascular (A), in the process of extravasating (B), or had extravasated (C) are shown at 3, 6, and 24 hr after injection. Different sets of experiments (involving different embryos) were performed at the stated time points. The data showed no significant differences among the three cell types, at any of the time points. By 24 hr after injection, >89% of observed cells of all three cell types had completed the process of extravasation. Bars represent means ± SE. (Republished from Koop *et al.*, 1996.)

Taken as a whole, the experiments reported in this and the previous section provide a strong indication that neither cancer cell arrest and survival in the microcirculation nor the process of extravasation into adjacent tissues



constitutes rate-limiting steps in the metastatic process. The reasons for metastatic inefficiency must therefore be sought at the level of postextravasation cell migration and growth.

## C. Postextravasation Migration of Cancer Cells

Extravasated cancer cells need to be able to grow at their new locations, and *in vivo* videomicroscopy has revealed that the cells may migrate short distances to preferred sites in host tissue prior to replication. The site of replication can differ depending on the tissue in which the cell has extravasated. In chick CAM, most of the extravasated cancer cells migrate through the mesenchyme and attach to arterioles, rather than to venules or lymphatics (Koop *et al.*, 1994, 1996). We have observed that the individual extravasated cells extend pseudopodial projections that wrap around the abluminal surfaces of vessels (Fig. 4c), where cells may later develop into tumors (Fig. 4d). The reason for this specific cell migration is currently unknown, but the same behavior was seen in chick CAM or mouse liver for every cell type we studied (melanoma and mammary carcinoma cell lines, *ras*-transformed fibroblasts) and was independent of metastatic potential or transformed status of the cells (Morris *et al.*, 1994, 1995; Koop *et al.*, 1994, 1996). These results suggest that directed migration might be of widespread significance. The migration toward the arterioles is not simply explained by a requirement for oxygen since the CAM is a respiratory organ, and thus the arterioles contain deoxygenated blood. Also no differences between the levels of fibronectin, laminin, or collagen have been detected in venules versus arterioles of the CAM (Ausprunk *et al.*, 1991). Thus, the molecular basis for directed migration remains to be determined. In the process of angiogenesis new blood vessels grow from host tissues into small tumors; however, our observations in the CAM show a complementary situation, in which extravasated cancer cells preferentially migrate to the vicinity of preexisting vessels (Koop *et al.*, 1994, 1996). We found that the cells did not begin to divide until after they had reached the arterioles (Fig. 4c) and tumors encircling the vessels were later found at these locations (Fig. 4d).

Postextravasation cell migration is also observed in mouse liver. After extravasation from sinusoids, the cells wrap around the abluminal surface of these vessels, in a manner similar to that seen in the CAM (Figs. 4a and 4c). However, cells that have extravasated from the most superficial sinusoids may send pseudopodial projections 20–30 μm long up to the subcapsular region of the liver (Fig. 3b), which, in the mouse, is an avascular region approximately 10 μm in thickness. Thereafter, migration of the cell body to this location occurs (Fig. 4b). Cells from both poorly and highly metastatic cell lines may be seen to have squeezed between hepatocytes at the liver surface, and in mouse

this is the primary location where hepatic metastases from many cancer cell lines develop. Pseudopodial projections similar to those exhibited by migrating cells *in vivo* (Fig. 3b) have also been observed during cell movement *in vitro* and found to contain membrane-bound proteinases, which facilitate cell movement by localized degradation of the matrix components (Kelly *et al.*, 1994; Monsky *et al.*, 1994). In addition, these projections contain $\beta_1$ integrins, which may also play a role in this cell movement (Nakahara *et al.*, 1996).

Recent studies have shown that integrin $\alpha_2\beta_1$ mediates postextravasation migration of human rhabdomyosarcoma and erythroleukemia cells in mouse liver but has no effect on their extravasation (Hangan *et al.*, 1996; Ho *et al.*, 1997). This result is in agreement with a model of diapedesis of leukocytes, which proposes that different integrins are involved in the different steps of extravasation and postextravasation cell movement (Weber and Springer, 1998). In this model the integrins $\alpha_4\beta_1$ and $\alpha_L\beta_2$ are involved in the transendothelial migration of the leukocytes and their movement through interendothelial cell junction, respectively, while $\alpha_5\beta_1$ is involved in the spreading and migration of the extravasated cells on the extracellular matrix (ECM) components. Thus cell migration *in vivo* is likely to be a very complex process that depends on the interaction of different molecules.

From our *in vivo* study of metastasis in chick CAM and mouse liver the following key concepts concerning postextravasation cell migration have emerged: (1) Following extravasation cancer cells do not appear to replicate until after they have migrated to specific sites in tissue. It is possible that these sites may contain substances that attract cells and presumably enhance tumor growth. (2) Extravasation and postextravasation cell movement appear to be distinctly different migratory processes, because in the cases we have examined each process depends on a separate molecular basis. (3) During cell movement *in vivo*, cells can undergo considerable deformation, stretching out long extensions (20–30 μm) through the tissue. These extensions could potentially allow cells to sample their environment and move through the tissue matrix to preferred sites for growth. Thus, the possibility exists that tumor metastasis could be suppressed by blocking the postextravasation migration of cancer cells.

## D. Postextravasation Cancer Cell Growth

Only a small proportion of cancer cells that enter the circulation from a primary tumor go on to form metastases, either clinically or in experimental animals (Liotta *et al.*, 1974; Butler and Gullino, 1975). Thus the metastatic process is "inefficient" (Weiss, 1983, 1990). Knowing where in the metastatic process this inefficiency occurs is of prime importance in devising strategies for preventing metastasis. In the organs we have examined, namely, chick CAM and mouse liver, and for the cancer cell lines we have utilized, the vast majority of injected cells (generally >80%) are able to survive in the microcirculation and have successfully extravasated by 1–3 days later. Therefore, the fact that the number of tumors produced is so small must mean that very few extravasated cells ever succeed in growing into tumors. The fate of extravasated cells is thus of primary importance in understanding metastatic inefficiency.



In the mouse liver model, we quantified the proportions of injected melanoma cells (B16F1) remaining at successive stages of the metastatic process (Luzzi *et al.*, 1998). The key findings are summarized in Fig. 6. The vast majority (>81%) of the injected cells remained as solitary extravasated cells at day 3; only 2% of the injected cells had started to divide and form micrometastases of 4–16 cells. (Our recent use of GFP-transfected cells now allows such micrometastases to be visualized with remarkable clarity by *in vivo* videomicroscopy; Figs. 3c and 3d). No larger colonies of cells were observed at this time. By day 13, only 1 in 100 of these micrometastases had gone on

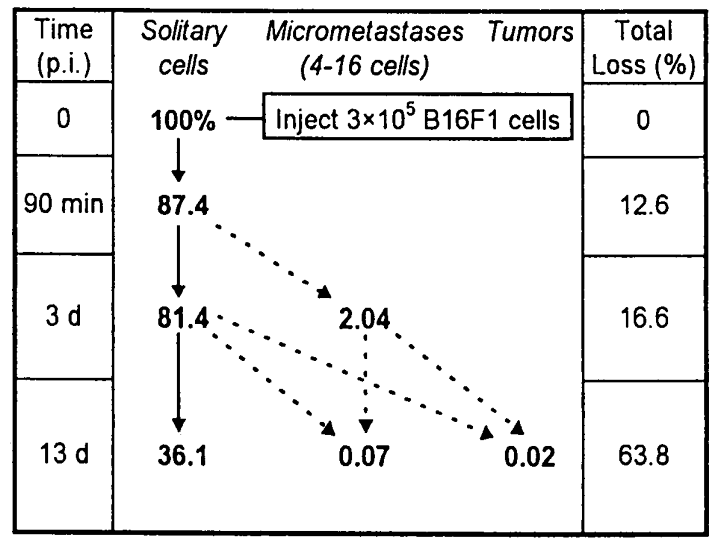


**Fig. 6** Flowchart summarizing survival data for B16F1 cells in mouse liver, showing the multistep nature of metastatic inefficiency. Percentages of injected cells remaining as solitary cells, or forming micrometastases or macroscopic tumors, at different times after injection (postinjection: p.i.) are shown. (At 90 min, >85% of injected cells were intravascular, whereas by 3 days >80% had completed extravasation.) Note the slow loss of solitary cells with time. Dotted arrows indicate possible origins of micrometastases and tumors. Two distinct steps after extravasation were principal determinants of metastatic inefficiency: the failure of solitary cells to initiate growth, and the failure of micrometastases to continue growth into macroscopic tumors. (Republished with permission from Luzzi *et al.*, 1998.)

to form tumors (similar to those seen in Fig. 3e) and the rest had disappeared; more than 36% of the injected cells still remained as solitary cells. This observation on solitary cells could result from a balance of proliferation and death resulting in a steady-state situation, or it could mean that these solitary cells are simply remaining dormant. We addressed this question using immunohistochemistry. The cells were stained in adjacent serial sections for markers for apoptosis (TUNEL) and proliferation (Ki-67). The identification of the melanoma cells was made using immunostaining for S100. Only 5% of the solitary cells were either undergoing proliferation or apoptosis. By comparison, 91% of the tumor cells in metastases were proliferating and 6% were undergoing apoptosis. These findings indicate that the solitary cells were in fact dormant. If these results also apply clinically, then these solitary, dormant cancer cells potentially pose a double threat to cancer patients. Dormant cells are not effectively treated by conventional cancer therapies that target only actively proliferating cancer cells. In addition, dormant cells could potentially be activated at a future time and start dividing to form metastases.

Our observations of extravasated cancer cells in mouse liver suggested that the main sources of metastatic inefficiency are found at two stages of tumor development. Only a very small fraction of solitary, extravasated cells (1 in 40 by day 3) began to divide to form micrometastases (4–16 cells). In addition, only a tiny fraction of these micrometastases (1 in 100) continued dividing to form tumors; most of the rest disappeared. These results suggest that initiation and maintenance of growth of micrometastases, as well as the activation of dormant, solitary cancer cells, could be important targets for future therapeutic strategies.

These concepts arise from our work on chick CAM and mouse liver concerning postextravasation cell growth: (1) A high proportion of extravasated cells can remain in the tissue for extended periods as solitary, dormant cells. (2) Metastatic inefficiency occurs as a two-step process with only a small proportion of extravasated cancer cells dividing to form micrometastases (4–16 cells). Only a tiny fraction of the micrometastases then go on to form tumors, while most of the rest disappear. Our results indicate that failure of extravasated cells to initiate growth in the target organ and failure of micrometastases to grow into macroscopic tumors may be two major rate-limiting steps in metastasis.

## V. TARGETS FOR ANTI-METASTASIS THERAPY: CLINICAL AND BIOLOGICAL CONSIDERATIONS

The goal for development of anti-metastatic therapeutics should be to prevent the negative physiologic consequences of growth of metastases to the patient. This goal can be met theoretically by a variety of strategies, targeted to specific steps in the process, from the prevention of the initial seeding of metastatic cells up to and including control of growth of established metastases (reviewed in Chambers, 1999; Fidler, 1999). This is a broader goal for cancer therapy than that taken by conventional cytotoxic treatment strategies (reviewed in Kohn and Liotta, 1995; Schipper *et al.*, 1996). Instead of requiring that all cancer cells be killed, this approach requires that the effects of the growth of the cells be minimized to the patient, by any strategy that achieves this end. Approaches to combating the physiologic effects of metastases are thus considered here in this context.



Three key questions must be addressed in developing new therapeutic approaches to combat the detrimental consequences of metastatic growth. First, what steps in metastasis offer appropriate targets, in terms of what is known about the *biology* of the metastatic process? Second, is there a *clinical* opportunity to apply a therapy designed to attack the chosen step? Third, are there *molecular* therapeutic approaches that can be developed to target the chosen step? Much of current research into anti-metastatic therapies is focused on this final question, with studies designed to determine, for example, how metastatic and nonmetastatic cells differ at the molecular level. Less attention is often paid to the first two questions, although answers to these questions are necessary in order to put molecular information about metastasis into a biological and clinical context. Careful consideration of biological and clinical factors that contribute to metastasis is required to determine how to use the molecular information that is rapidly accumulating about metastasis, as well as to prevent unnecessary effort being applied to inappropriate therapeutic targets. Here we focus our attention on lessons learned from biological studies on the metastatic process, as outlined earlier, coupled with clinical considerations as to the accessibility of biologically promising therapeutic targets in the natural history of cancer and metastasis in patients.

### A. Clinical and Biological Relevance of Specific Steps in Metastasis as Therapeutic Targets

A list of steps that occur during the metastatic process is shown in Fig. 7, beginning with growth of the primary tumor and culminating with growth of the metastatic lesions. For each step in the process, two questions need to be answered: *Is this step a promising therapeutic target, based on what is known about the biology of the process?* and *Is this step a promising therapeutic target, based on the clinical opportunity for applying a therapy targeted to that step?* Only then can one profitably ask whether a drug or other therapeutic approach is available, or can be developed, to target the step in question.

Ideally, one would like to target and prevent the earliest steps possible in the metastatic process, by preventing the *growth of the primary tumor* (see

Fig. 7). Biologically, this step is clearly an appropriate target, and would essentially short circuit the whole metastatic process. Clinically, however, the opportunity to target the growth of the primary tumor is relatively limited, once a patient has been identified as having cancer. The goal for many cancers is to remove the primary tumor as promptly as possible, and this can often be achieved successfully with surgery. The time window for targeting growth of the primary tumor with the aim of preventing metastasis is thus relatively limited. In addition, by the time a tumor has been detected clinically, it may already have released metastatic cells that have progressed through several of the subsequent steps. In this situation, there might be little value in trying to prevent further growth of the primary tumor. In the future, however, this early phase of tumor progression may well be amenable to chemoprevention strategies, especially in patients deemed to be at higher risk for developing subsequent cancer. An example of this strategy is the recent Tamoxifen prevention trial for women at high risk for developing breast cancer (Fisher *et al.*, 1998). Similarly, dietary and lifestyle interventions designed to prevent cancer development in people who are not identified as already having cancer (reviewed by Schatzkin, 1997) would ultimately, if successful, have the effect of preventing metastasis at the very earliest phase of the process.

| Steps in metastasis | Good Therapeutic Target: Clinically? | Biologically? |
|---|---|---|
| • Growth of 1° tumor | • Limited opportunity, after diagnosis of 1° tumor<br>• Possibly useful in chemoprevention setting? | • Good biological target |
| • Intravasation | • Limited opportunity, after diagnosis of 1° tumor | • Insufficient evidence |
| • Survival in circulation | • Unlikely | • Unlikely |
| • Arrest in new organ | • Unlikely | • Unlikely |
| • Extravasation | • Unlikely | • Unlikely |
| • Initiation of growth/ dormant cells | • Promising clinical target | • Promising biological target |
| • Persistence of growth/ angiogenesis | • Promising clinical target | • Promising biological target |

**Fig. 7** Steps in the metastatic process and assessment of whether each step offers a good therapeutic target, based on biological and clinical considerations.



Relatively little is known biologically or clinically about the *intravasation* process (the escape of cancer cells from the primary tumor to the blood or lymphatic circulation), making it difficult to know if this stage is an appropriate one for intervention (Fig. 7). Few models have been designed for studying the intravasation process by direct experimentation (e.g., see Kim *et al.*, 1998). Clinically, little can be known about the timing or extent of intravasation, for a given tumor. The consequences of intravasation are used clinically in, for example, detection of breast cancer cells in a patient's lymph nodes, as a marker of cells having begun to be shed from the tumor, and the presence of these cells is an indicator of poor prognosis in breast cancer. However, considerably more needs to be learned about the intravasation process, both biologically and clinically, before it can be determined if this step in metastasis offers a promising therapeutic target.

The steps in the metastatic process that include cancer cell *survival in the circulation, arrest in a new organ,* and *extravasation* from the circulation into the tissue of the new organ may be less than optimal steps for the development of anti-metastatic therapies (Fig. 7), for several reasons. First, our biological studies have indicated that cancer cells do not appear to be particularly vulnerable to attack at these stages, and that cells can successfully complete these steps in large numbers, as discussed earlier. In addition, these steps appear to be relatively independent of the malignant phenotype of the cells, with highly and poorly metastatic cells being similarly able to complete these steps. Furthermore, these steps appear to be completed quite rapidly, thus providing a very restricted temporal target. We have found, for a variety of cancer cell types injected to target to several different organs, that the vast majority of a population of circulating cells has completed the process of lodging in an organ and fully extravasating within 1–3 days of being injected into the circulation. For an individual cancer cell, the process of arrest and extravasation appears to require only hours to complete. Thus, these steps appear to be targets of limited utility in terms of the biology of the process.

Similarly, these steps (survival in the circulation, arrest and extravasation) may not be ideal targets in terms of the clinical course of the disease (Fig. 7). In a patient who has just been diagnosed with cancer, there would be a limited temporal window for treatment to prevent these steps in metastasis. If the primary tumor has progressed to the point of having shed cells into the circulation, the majority of those would already have arrested and extravasated in secondary sites, and only cells shed from the primary during the relatively brief time from diagnosis to initial treatment would be vulnerable to treatment of these steps. It is also possible that there may be a window of therapeutic opportunity at the time of primary surgery for cancer, if

appreciable numbers of cancer cells are shed at the time of surgery (discussed in Chambers, 1999). In general, however, this stage of the process seems to be of limited promise as a target, based on both biological and clinical considerations.

A much broader time window is offered by the final steps in the metastatic process, the *growth of metastases in secondary sites* (Fig. 7). Included in this process are the initiation of growth of extravasated cells (or the maintenance of dormancy in a population of single cells), the persistence of growth of pre-angiogenic metastases, and the attraction of new vasculature to support continued growth of metastases. Biologically, this phase of metastasis appears to offer a very promising and multifaceted therapeutic target. Our *in vivo* studies described earlier have consistently pointed to the growth phase, after cancer cells have successfully extravasated in secondary sites, as being key in the regulation of metastasis.

Clinically, the growth phase of the metastatic process also appears to offer an excellent target, since the restriction of growth at any point prior to the metastases causing irreversible physiologic harm to the patient has the potential to be clinically useful. During the clinical course of the disease, this phase occupies a much larger proportion of time than do the steps of arrest in the circulation and extravasation into the tissue.

## B. Therapeutic Approaches for Restricting Growth of Metastases

Based on both biological and clinical considerations, it thus appears that the restriction of growth of metastases in secondary sites may offer an especially promising target for development of therapeutic strategies to combat the consequences of metastasis to the patient (Fig. 7). There are a number of approaches by which this could be achieved, and focused attention of research into this area seems warranted. Current cytotoxic chemotherapy is indeed directed at this general target, but is based primarily on the idea that cells populating the metastases need to be dividing rapidly in order to be killed. As is well known, the side effects of these therapies to the patient are based on the nonspecificity of this requirement, and many normal tissues are equally or more sensitive to the cytotoxic effects of this approach. Alternate strategies, based more on growth pathways or other aspects of growth with greater specificity for the cancer cells, are needed (Kohn and Liotta, 1995; Schipper *et al.*, 1996). A number of such strategies are under active development, and an understanding of biological and clinical factors in metastasis will be important for appropriate use of these approaches. Although details of these strategies are beyond the scope of this review, some promising means to achieve this end are mentioned here.



One approach that might lead to growth inhibition of metastases is through *inhibition of signal transduction pathways* used by metastatic tumors to support their growth. Considerable effort is devoted to identifying unique, or preferential, signaling pathways used by malignant cells, and to the development of inhibitors of these pathways. One example of this approach is the development of the cytostatic drug carboxyamidotriazole (CAI), an inhibitor of calcium-mediated signal transduction pathways (Kohn *et al.*, 1992). Interestingly, CAI has been shown to affect growth of tumor cells directly (Kohn *et al.*, 1994) as well as indirectly via an inhibition of metastasis-specific angiogenesis (Luzzi *et al.*, 1999). Another example of the strategy to limit growth of metastases via inhibition of signaling pathways active in malignant cells is the use of anti-HER2/neu monoclonal antibody in women with HER2/neu-positive breast tumors (M. D. Pegram *et al.*, 1998, 1999; Norton, 1999).

Another attractive approach to limiting growth of metastasis is through the inhibition of *angiogenesis,* the development of new blood vessels which are required for persistence of growth of tumors. By limiting the development of new vasculature, metastases would be restricted to small sizes, which are generally of minimal clinical consequences to the patient. This approach is particularly promising in that it is both biologically sound and there would be clinical opportunity for applying this strategy (both for restricting the growth of metastases, whether clinically detected or occult, as well as inoperable primary tumors). This strategy is the subject of considerable attention and research (reviewed by Folkman, 1995, 1996; Pluda, 1997; Zetter, 1998).

## C. "Seed" and "Soil" Revisited: Organ-Specific Growth as an Anti-Metastasis Therapeutic Target

It has long been observed that certain tumors show an organ-specific pattern of metastasis. Breast cancer, for example, preferentially metastasizes to bone, liver, brain, and lung. In 1889, Stephen Paget published an article in the *Lancet* entitled "The Distribution of Secondary Growths in Cancer of the Breast" (see also Poste and Paruch, 1989). In his paper, Paget conceptually addressed the question "What is it that decides what organs shall suffer in a case of disseminated cancer?" Paget's conclusion can be summarized by the following quote from his article: "When a plant goes to seed, its seeds are carried in all directions; but they can only live and grow if they fall on congenial soil." Where metastases finally form is a relatively straightforward question, which can be addressed by looking at the "endpoint" of metastasis, that is, the presence or absence of detectable metastases in specific organs. The question of whether cells from a primary tumor are carried "everywhere" in the body is a much more difficult question to ask.

The concept that certain tumors "go" to specific target organs thus should more properly be replaced with the concept that certain tumors "grow" in specific target organs, since the endpoint of detectable tumors gives evidence only of successful growth, and no indication of whether cells arrived in the organ but failed to grow there. Our experimental *in vivo* studies, described earlier, have suggested that the majority of cells injected to target them to a specific organ in fact arrest and successfully extravasate in that organ, with the initial arrest in the microcirculation being determined primarily by size considerations. Cells from most solid tumors are large, relative to the sizes of most capillaries, although there are size differences both between cancer cell types and between capillaries in different organs. A small proportion of cells may pass through the first microcirculatory bed encountered and on to the next. Consideration of blood flow patterns predict to which organs cells shed from tumor growing in specific organs will be carried first (see Chambers *et al.*, 1995, Fig. 3 therein). For example, cancer cells shed from a primary tumor in a splanchnic organ such as the gastrointestinal tract or pancreas will first be taken by the hepatic portal circulatory system to the liver. Cells shed from a lung tumor would be taken via the arterial system to all parts of the body. Cells from tumors in other organs would be transported via the venous system first to lung capillaries. Patterns of lymphatic drainage of specific organs also are important, and cancer cells can be spread in this fashion as well.

A detailed study of autopsy data, coupled with considerations of relative blood flow to specific organs, was conducted by Weiss (1992). In that study, the presence of metastases detected at autopsy of cancer patients was used to determine the pattern of metastatic spread. Arterial blood flow to specific organs (in ml/min) was used to determine exposure of the organ to circulating cancer cells; arterial flow only was considered, and metastases to liver and lung were excluded. Based on this analysis, Weiss concluded that metastasis to most organs can be accounted for primarily by the relative volumes of blood delivered to the organ. However, some organs were deemed to be "hostile" or "friendly" in their support of growth of cancer cells delivered to the organ. For example, breast and prostate cancers showed preferential growth in bone, above that which would be expected on the basis of volume of blood flow to the bone. This study thus suggests that much of organ-specific metastasis may be due to the volume of blood passing through various target organs. However, several factors could not be assessed with this sort of approach, including numbers of cells shed from different primary tumors, patterns of blood flow between primary and secondary sites, and, importantly, numbers of cells carried to secondary sites. The relationship between numbers of cancer cells that arrest (most of which likely survive and extravasate, as discussed earlier) in an organ, and the formation of detectable metastases in that organ (i.e., organ-specific metastatic inefficiency), is important for a full understanding of organ-specific metastasis.



Based on the considerations mentioned, the important factors in determining organ specificity of metastasis can be summarized as in Fig. 8. This topic has been recently reviewed by Radinsky (1995) and Fidler (1995). Two factors determine the distribution for specific tumor types: the number of cells that arrest in an organ, and the ability of the environment of that organ to support growth of the cells. Metastasis clearly involves interactions between cancer cells (the "seeds"), and specific organs and microenvironments within organs (the "soil"). An improved understanding of the factors that contribute to organ-specific growth promotion or inhibition will be important for the development of anti-metastasis therapies.

Two features of organ-specific metastatic growth are important: First, what factors influence the preferential growth of certain types of cancer cells in specific organs? (That is, why do breast cancer cells preferentially grow in bone?) Second, what factors influence the selective growth of a small subset of a cancer cell population that arrives in an organ? (That is, why do only subsets of breast cancer cells begin and sustain growth in bone, while other

| Organ-Specific Metastasis | |
|---|---|
| ① Cancer cells are 'seeded' wherever the circulation takes them | • Circulatory patterns from the primary tumor<br>• Size of cancer cells relative to capillaries in different organs |
| ② Cancer cells grow only where they find a supportive growth environment | • 'Seed' (cancer cell) specific factors *(e.g. oncogenes, receptors, signal transduction pathways that are active, etc.)*<br>• 'Soil' (organ) specific factors *(e.g. growth factors, extracellular matrix components, etc.)* |

**Fig. 8** Organ-specific metastasis. Two steps in organ-specific metastasis are listed, along with factors that contribute to each of these steps.

cells from the same breast tumor fail to grow? Are there differences within subpopulations of cancer cells, differences in microenvironment where the cells find themselves by chance, or a combination of both?) Clarification of these questions will add to our ability to target growth of metastases as an anti-metastasis therapeutic strategy.

## VI. CONCLUSIONS

Metastasis is responsible for the majority of deaths due to cancer. Therapeutic strategies that effectively prevent the development and growth of metastases thus have the potential to impact on cancer mortality. Outlined in this article are biological considerations of the metastatic process, with a focus on information learned from *in vivo* videomicroscopy studies of the metastatic process, coupled with techniques to quantify the loss of cells at various steps in the process. These studies have led to the conclusion that the most promising targets for development of anti-metastasis therapies lie in the growth phase of the process, after cancer cells have arrived in secondary sites and extravasated there. This phase of the metastatic process is also attractive clinically, providing a broad time window for treatment, because clinical utility is possible at any point prior to growth of metastases causing irreversible clinical consequences to the patient. Earlier phases of the metastatic process, such as survival of cells in the circulation, arrest in secondary sites, and extravasation, appear to be less appropriate as therapeutic targets, based both on biological and clinical considerations. Approaches being developed to target the growth phase of metastasis include inhibition of angiogenesis of metastatic tumors, and inhibition of signaling pathways required by metastases for their growth.

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